ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF HYDROCARBON DEGRADING BACTERIA FROM SOIL CONTAMINATED WITH USED ENGINE OIL

BY

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CERTIFICATION

This is to certify that this project work titled; Isolation and Biochemical Characterization of Hydrocarbon Degrading Bacteria from Soil Contaminated with Used Engine Oil was written and carried out by **KOMOLAFE, HELLEN IDOWU** with matric number **MCB/14/2325**, a student of the Department of Microbiology, Faculty of science, Federal university Oye-Ekiti, Ekiti state, Nigeria.

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DEDICATION

This research work is dedicated to God Almighty, who began this journey with me and saw me through: he is the only wise God.

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ABSTRACT

The oils containing hydrocarbon such as crude oil, petrol, engine oil, diesel etc., causes environmental pollution. Hydrocarbon degrading bacteria use hydrocarbon for their growth and metabolic activities. The soil samples were collected from four different mechanic workshops in Oye-Ekiti and Ilupeju-Ekiti. The bacteria capable of degrading hydrocarbons present in the soil were isolated using Bushnell Haas agar with used engine oil as the sole carbon source. A total number of 6 bacterial isolates were identified in this study based on their cultural, morphological and biochemical characteristics. The identified bacteria were *Staphylococcus aureus*, *Alcaligenes* sp., *Pseudomonas* sp., *Staphylococcus epidermidis*, *Bacillus* sp. *and Micrococcus* sp. The bacterial growth rate was determined by measuring the optical density using the spectrophotometer at wavelength 600nm and also by colony count from day 0 to day 4. The result revealed that *Pseudomonas* sp. and *Micrococcus* sp. had the highest number of colonies (171-360 & 150-390 respectively) and highest optical density (0.152A-0.335A & 0.203A-0.368A respectively). The effect of different carbon sources, nitrogen sources and pH on the growth of *Pseudomonas* sp. and *Micrococcus* sp. was determined by measuring the optical density using the spectrophotometer at wavelength 600nm and also by colony count from day 0 to day 4. The carbon sources used were used engine oil, diesel and kerosene. The result revealed that the best carbon source for the growth of *Pseudomonas* sp. and *Micrococcus* sp. was used engine oil. *Pseudomonas* sp. was also able to utilize kerosene more than *Micrococcus* sp. The nitrogen sources used were ammonium nitrate, ammonium sulphate and ammonium chloride. The result revealed that both *Pseudomonas* sp. and *Micrococcus* sp. was able to utilize ammonium nitrate as the best nitrogen source compared to ammonium sulphate and ammonium chloride. The pH used were 6, 7, and 8. The result revealed that both *Pseudomonas* sp. and *Micrococcus* sp. was able to grow best at pH 7.

CHAPTER ONE

1.0. INTRODUCTION

Since the advent of oil exploration, the Nigeria environment has been heavily contaminated with hydrocarbon pollutants, which enters the environment through several route. The presence of these pollutants in the terrestrial and aquatic environments constitutes public health and socio-economic hazards (Edewor *et al.*, 2004; Jain *et al.*, 2011; Okerentugba and Ezeronye, 2003).

Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents that is used to lubricate the parts of an automobiles engine, in order to keep everything running smoothly (Adelowo and Oloke, 2002). The most important characteristic of the lubricating oil for automotive use is its viscosity. New motor oil contains a higher percentage of fresh and lighter (often more volatile and water soluble) hydrocarbons that would be more of a concern for acute toxicity to organisms (Albaiges *et al.*, 2006). Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Boonchan *et al.*, 2000; Albaiges *et al.*, 2006).

In Nigeria, it is common among motor mechanics to dispose spent engine oil into gutters, water drains and soil (Okonokhua *et al.*, 2007; Islas-Garcia *et al.*, 2015). Spent engine oil is defined as used lubricating oils obtained after servicing and subsequently draining from automobile and generator engines. Spent oils contain high percentage of aromatic and aliphatic hydrocarbons, nitrogen and sulphur compounds and metals (Mg, Ca, Zn, Pb) than fresh oils, these metals are introduced into the oil as a result of wear and tear of the engine (Mohd *et al.*, 2011).

Spent engine oil causes great damage to soil and soil microflora. It creates unsatisfactory condition for life in the soil due to poor aeration, immobilization of soil nutrients and lowering of soil pH, loss of soil fertility (Ugoh and Moneke, 2011). It has been shown that marked changes in properties occur in soil contaminated with hydrocarbon; this affects the physical, chemical and microbiological properties of the soil (Okonokhua *et al.*, 2007). At low concentrations, some of these heavy metals are essential micronutrients for plants, but they can cause metabolic disorders and growth inhibition when the concentration is high. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al.*, 2001)

In addition, PAHs have a widespread occurrence in various ecosystems that contribute to the persistence of these compounds in the environment. The illegal dumping of used motor oil is an environmental hazard globally (Van Hamme *et al.*, 2003). The release of oil into the environment causes environmental concern and attracts the public attention (Roling *et al.*, 2002). Physiochemical or Mechanical method to reduce hydrocarbon pollution is expensive and time consuming and often involve the rise of spreading the pollution because the waste would require disposal elsewhere. Hydrocarbons including PAHs have been long recognized as substrates supporting microbial growth, therefore a better way would be to use bioremediation (Edewor *et al.*, 2004).

Bioremediation method is considered to be more economical and safe method for the treatment of oil contaminated site. It has been observed that microorganisms that grow on oil contaminated soil are much capable of degrading oil than those microorganisms which are found on non-contaminated site of oil (Harder, 2004). Bioremediation makes use of indigenous oil–consuming microorganisms, called petrophiles, by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons and utilize them as a food source. Microorganisms degrade these compounds by

using enzymes in their metabolism and can be useful in cleaning up contaminated sites (Milic *et al.*, 2009). Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These microorganisms can degrade a wide range of target constituents present in oily sludge (Barathi and Vasudevan, 2001; Mishra *et al.*, 2001).

A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers. Other petroleum hydrocarbon-degraders include *Yokenella spp., Alcaligenes spp., Roseomonas spp., Stenotrophomonas spp., Acinetobacter spp., Flavobacter spp., Corynebacterium spp., Streptococcus spp., Providencia spp., Sphingobacterium spp., Capnocytophaga spp., Moraxella spp., and Bacillus spp.* (Bhattacharya *et al.*, 2002). Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent. However, they take longer periods of time to grow as compared to their bacterial counterparts (Prenafeta-Boldu *et al.*, 2001).

Bioremediation processes have been shown to be effective methods that stimulate the biodegradation in contaminated soils (McLaughlin, 2001). Harder, (2004) estimated that bioremediation accounts for 5 to 10 percent of all pollution treatment and has been used successfully in cleaning up the illegal dumping of used engine oil. This study was therefore carried out to isolate potential hydrocarbon-degraders in soil samples contaminated with used engine oil and to determine the optimum conditions required for the growth of these isolates.

1.1. JUSTIFICATION

Hydrocarbon compounds such as petroleum although occur in the forms useful to humans, they can be hazardous. Fuel and lubricating oil spills have become a major environmental hazard to date (Van Hamme *et al.*, 2003). The release of petroleum hydrocarbon into the environment result into serious problems to humans, ecosystem, animals and plants. Microorganisms has

been found to clean up or degrade hydrocarbon (Mishra *et al.,* 2001). Hence, there is need to isolate and identify the organisms that degrade hydrocarbon.

1.2. AIM & OBJECTIVES

1.2.1. AIM

To isolate and identify petroleum hydrocarbon degraders in soil contaminated with used engine oil.

1.2.2. OBJECTIVES

- Isolation of microorganisms from soils contaminated with used engine oil;
- Identification of isolates by means of cultural, morphological, and biochemical characteristics tests;
- Optimization of growth conditions (carbon sources, nitrogen sources and pH) to enhance the growth of hydrocarbon degrading bacteria.

CHAPTER TWO

2.0. LITERATURE REVIEW

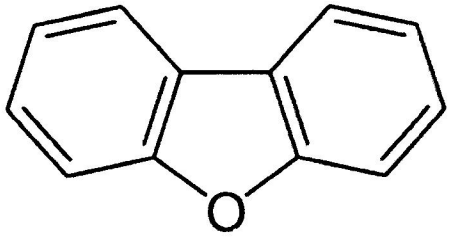
2.1. Petroleum hydrocarbon

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo-constituents. Petroleum constituents represent: saturates, aromatics, resins and asphaltenes. Saturates refer to hydrocarbons containing no double bonds (Harayama, 2004). They are categorized according to their chemical structures into alkanes (paraffins) and cycloalkanes. Saturates represents the highest percentage of crude oil constituents. Aromatic hydrocarbons having one or several aromatic rings are usually substituted with different alkyl groups. In comparison to the saturated and aromatic fractions, the resin and asphaltenes contain non-hydrocarbon polar compounds. Resins and asphaltenes have very complex and mostly unknown carbon structure with addition of many nitrogen, sulphur and oxygen atoms (Harayama, 2004). Petroleum products are used as fuels, solvents and feedstock in the textile, pharmaceutical and plastic industries

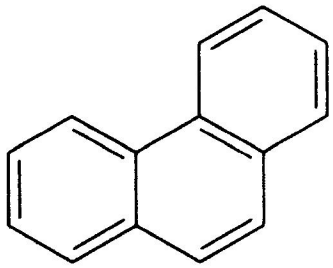
Petroleum recovered from different reservoirs varies widely in compositional and physical properties. The composition of particular petroleum product ranges from the very low molecular weight hydrocarbons to the very high molecular weight ones. A hydrocarbon's chemical structure affects its biodegradation in two ways. First, the molecule may contain groups or substituents that cannot react with available or inducible enzymes (Riser-Roberts, 1992). Second, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur. Usually, the larger and more complex the structure of a hydrocarbon, the more slowly it is oxidized. Also the degree of substitution affects the degradation. Compounds that contain amine, methoxyl and sulphonate groups, ether linkages,

halogens and branched carbon chains are generally persistent. Adding aliphatic side -chains increases the susceptibility of cyclic hydrocarbons to microbial attack (Riser-Roberts, 1992).

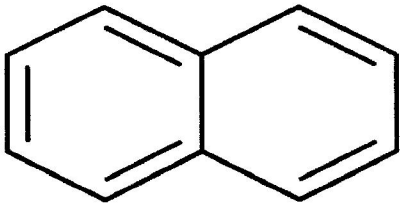
Hydrocarbon composition affects the physicochemical properties of microorganism (Carvalho and Fonseca, 2005). Hydrocarbons differ in their solubility, from polar compounds, such as methanol to very low solubility nonpolar compounds, such as high molecular weight polynuclear aromatic hydrocarbons (Riser-Roberts, 1992). The solubilization is not the only factor determining the degradation of hydrocarbons. Many microorganisms, such as *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus laterospor* excrete emulsifiers that increase the surface area of the substrate. On the other hand, these microorganisms modify their cell surface to increase its affinity for hydrophobic substrates and, thus facilitate their absorption (Carvalho and Fonseca, 2005). Hydrocarbons can be very fluid or very viscous and very volatile or relative non-volatile. Viscosity of polluting oils is an important property. It determines the spreading and dispersion of the hydrocarbon mixture and also the surface area available for microbial attack. The variability in the physicochemical character of hydrocarbons causes changes in the behaviour of individual hydrocarbons as well as mixtures (Cybulski *et al.*, 2003).



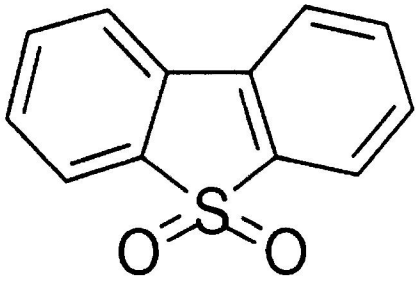
Dibenzofuran



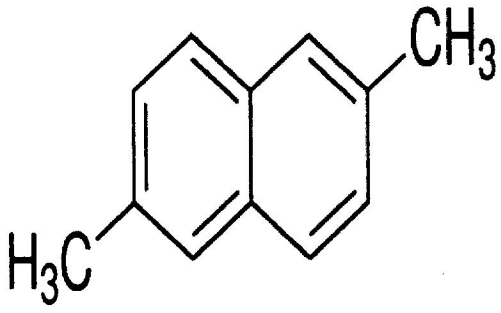
Phenathrene



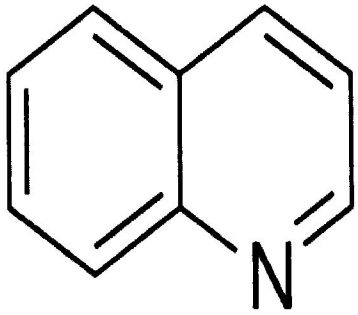
Naphthalene



Dibenzothiophene



2,6-dimethylnaphthalene



Quinoline

Figure 1: Structure of some polycyclic aromatic hydrocarbons (Shukla and Cameotra, 2012)

2.2. Effect of petroleum hydrocarbon contamination on living things and the environment

Living matter can be exposed to petroleum in so many ways either directly or indirectly. Some by-products formed during petroleum refining and processing which are used for the

manufacturing of other products are highly toxic. The highly toxic chemicals contained in crude oil can damage some organ systems in the human like the nervous system, respiratory system, circulatory system, immune system, reproductive system, sensory system, endocrine system, liver, kidney, etc. and thereby cause a wide range of diseases and disorders (Mahatnirunkul *et al.*, 2002). Individuals more susceptible to harm by the toxic effects of crude oil include:

1. Infants, children, and unborn babies.
2. Pregnant women.
3. People with pre-existing serious health problems.
4. People living in conditions that impose health stress.

Singh *et al.* (2004), studied the toxicity of fuels of different chemical composition on CD-1 mice (A Swiss mice strain is used as a general purpose stock and an oncological and pharmaceutical research. This is a vigorous outbred stock. These mice are fairly docile and easy to handle). The objective of the study was to establish a correlation between the physico-chemical properties of the fuel and how they affect the mice i.e. their biologic effects on the mice. The results of the study illustrated that the automobile derived diesel exhaust particles were more toxic than the exhaust generated by forklift engines. It was also found that the diesel exhaust particles contain ten times more extractable organic matter than the standard exhaust material generated by forklift engines.

A similar study was conducted by Kinawy (2009), which revealed that the inhalation of leaded or unleaded (containing aromatics and oxygenated compounds) gasoline vapours by rats impaired the levels of monoamine neurotransmitters and other biochemical parameters in different areas of the rats' brains. Likewise, several behavioural changes causing aggression in rats were observed.

Menkes and Fawcett (1997), discussed the toxicities of lead and manganese added gasoline and the public health hazards due to aromatic and oxygenated compounds in gasoline. The extent of absorption of petroleum components by inhalation, oral, and dermal routes varies significantly because of the wide range of physicochemical properties of these components. The incorporation of crude oil into the body may affect the reproductive health of humans and to other lives.

Okecha (2000), reported that crude oil changes the characteristics of soil, polluting it thereby becoming harmful to living organisms. He also stated that vegetation, wildlife, crops and farmlands are widely affected. Onwurah (2000), also reported that polycyclic aromatic hydrocarbons (a derivative of crude oil) can be carcinogenic and can lead to the rapid death of living organisms.

Obidike *et al.* (2007), observed that when the male rats were given an oral crude oil treatment using a drenching tube, degeneration and necrosis of interstitial cell occurred followed by the exudation into the interstices in the testes of rats. The study concluded that exposure of rats to crude oil induces reproductive cytotoxicity confined to the differentiating spermatogonia compartment, likewise it may also harm human reproductive cells. The extent of absorption through the various routes depends on the volatility, solubility, and other properties of the specific component or mixture. The more volatile and soluble the oil fractions (low molecular weight aliphatic and light aromatic compounds) are the faster they can leak into groundwater or vaporize into the air.

Table 1: Hazardous effect of petroleum hydrocarbon on environment

Parameters	Hazardous Effect	Reference
Agriculture	□ Soil fertility reduces □ Affects physiological properties of soil □ Has adverse effect on seed germination	Yoshida *et al.* (2006), Gong *et al.* (2001), Wyszkowska and Kucharski, (2000)
Aquatic life	□ Death of natural flora and fauna (oil causes anaerobic condition) □ Aquatic birds suffer from hypothermia, drowning, loss in flight, poisoning □ Reproductive impairment in fish	Carneiro *et al.* (2010), Torres *et al.* (2008), Gelin *et al.* (2003), Peterson, (2001)
Human	□ Severe disease (skin erythema, skin cancer, sinonasal cancer and bladder cancer) □ Effect on CNS □ Depression □ Irregular heartbeat	Lewis *et al.* (2008), Chen *et al.* (2008), Rice *et al.* (2007), Lee *et al.* (2006)

Ecosystem	□ Imbalance in marine ecosystem □ Physical and chemical alteration of natural habitats □ Imbalance in food chain	Carneiro *et al.* (2010), Torres *et al.* (2008), Gelin *et al.* (2003), Peterson, (2001)
Plants	□ Plants covered with oil are unable to photosynthesize	Gelin *et al.* (2003), Carneiro *et al.* (2010), Wrabel and peckol, (2000)
Animals	□ Crude oil exposure may cause damage to lungs, liver, kidney, intestine and other internal organs	Knafla *et al.* (2006), Lewis *et al.* (2008), Rice *et al.* (2007)

2.3. Methods used for cleaning up of petroleum hydrocarbon from contaminated soil

2.3.1. Physio-chemical method

Soil excavation is the mechanical removal of contaminated soils from sites of contamination either for burying or burning. Araruna *et al.* (2004), reported the disadvantage of the process. He stated that the method is expensive due to the fact that a contractor has to be hired to take away a layer of ground. He also stated that the layer from which the soil is removed is prone to erosion and other environment damaging agent.

Anderson (1993), reported that soil washing is an ex situ treatment process applicable to a broad range of organic, inorganic, and radioactive contaminants in soil. According to Wood (2092), soil washing is a method that involves the use of liquid/ water sometimes combined with chemical additives and a mechanical instrument to scrub soil. He also stated that this method removes hazardous contaminants and concentrates them into smaller volumes. Wood

(2002). also stated that hazardous chemicals easily adhere to silt and clay unlike sand and gravel particles and that during soil washing, the silt and clay are mechanically separated from the uncontaminated coarse soils. He also reported that the contaminated fine sand can then be disposed or treated accordingly while the coarse sand is retained as backfill. He also stated that the effectiveness of this method has been shown to be less than 80% though efficiency increases when hot water is used. It is therefore mostly used as a pre-treatment method for final cleaning up of soils.

Cole (1994), described soil vapour extraction as a method that involve the use of a specially designed system to remove volatile contaminants e.g. Crude oil from soil in form of vapour. Imamura *et al.* (1997), stated that the process of soil vapour extraction is carried out by applying a vacuum through a system of underground wells which pulls up contaminants to the surface as vapour or gas. He also stated that air is sometimes introduced to enhance the process. Soil vapour extraction is frequently used to remove chlorinated hydrocarbons. especially trichloroethylene (TCE) from soil.

2.3.2. Thermal method

Incineration, which involve burning off the contaminants from the soil surface using fire. According to USEPA (2000), incineration takes place at high temperature (i.e. between 1,600°F and 2,500°F) and hazardous wastes including crude oil are destroyed from the soil and toxic elements are reduced to basic elements (mainly hydrogen, carbon, chlorine and nitrogen). The basic elements then combine with oxygen to form stable non-toxic substances such as water, carbondioxide and nitrogen oxides. Bassam and Battikhi (2005), stated that contaminated soils are normally first excavated and carried to off-site facilities before incineration is carried out. Araruna *et al.* (2004), Bassam and Battikhi (2005), reported the

disadvantages of incineration to include: High operational cost due to high energy requirement, the large space involved and the dangers of environmental pollution.

Another method is thermal desorption. Elgibaly (1999), described thermal desorption as a method that involve heating up crude oil contaminated soils to temperature of 200-1000°F at which contaminates with low boiling point vaporize and desorb (i.e. physical separation) from the soil. It can also be termed as Low Temperature Thermal Desorption or Low Temperature Thermal Volatilization due to its use of low temperature. Anderson (1993), also referred to this method as thermal stripping or soil roasting. Wood (2002), reported that mostly during thermal desorption, contaminating hydrocarbons are vaporized and ignited. The remaining by-product are removed from the system by convection and treated by filters or second stage re-ignition or by an air emission treatment system. Up to 90% efficiency has been recorded with thermal desorption in removal of crude oil hydrocarbon contaminants from soils. Wood (2002), also stated that thermal desorption has three major pitfalls/disadvantages: It is expensive, time consuming and hazardous. Elgibaly (1999), stated on the other hand that thermal method seems to be a very promising method for cleaning contaminated soil because it is simple and avoids digging of soil which can be difficult.

2.3.3. Biological method

One of the biological method used is bioremediation, which is the process of digestion, assimilation and hydrocarbon metabolism carried out by bacteria, fungi, protozoa and other organisms. The process of biodegradation includes the following reactions: oxidation-reduction, adsorption processes and ion exchange, as well as chelation reactions that result in metal build-up. Microbial biodegradability allows the transformation of hydrocarbons of complex structure into a more simple chemical structure (Widel *et al.*, 2001). Kamada *et al.* (2002) and Madigan *et al.* (2003), stated that when the transformation is simple, it is called

"primary", when complete, "mineralization", in the latter, hydrocarbon is decomposed into inorganic compounds and/or cellular constituents. Xu and Obbard (2004), reported that the acceleration in hydrocarbon biodegradation may be carried out by native microorganisms or bio-augmentation, with appropriate nutritional and environmental conditions.

Phytoremediation is an emerging technology that uses plants to manage a wide variety of environmental pollution problems, including the clean-up of soils and groundwater contaminated with hydrocarbons and other hazardous substances. According to USEPA (2000), the different mechanisms that could be utilized for the remediation of a wide variety of contaminants include, hydraulic control, phytovolatilization, rhizoremediation, and phytotransformation. The advantages of using phytoremediation as reported by Khan *et al.* (2000), include cost effectiveness, aesthetic advantages, and long-term applicability. Furthermore, he stated that the use of phytoremediation as a secondary or polishing *in situ* treatment step minimizes land disturbance and eliminates transportation and liability costs associated with offsite treatment and disposal.

2.4. Microbial degradation of petroleum hydrocarbon

Microorganisms that carry out biodegradation in many different environments are identified as active members of microbial consortiums. Microorganisms individually cannot mineralize most hazardous compounds. Complete mineralization results in a sequential degradation by a consortium of microorganisms and involves synergism and co-metabolism actions (Chaillan *et al.*, 2004).

Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye *et al.* (2007). Nine bacterial strains, which include, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and*

Corynebacterium sp. were isolated from the polluted stream which was found to degrade crude oil.

Bacteria are the most active agents in petroleum degradation, they as well work as primary degraders of petroleum hydrocarbon in environments (Rahman *et al.*, 2003, Brooijmans *et al.*, 2009). Several bacteria are even known to feed solely on hydrocarbons (Yakimov *et al.*, 2007). Bacterial genera, namely, *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia*, and *Mycobacterium* isolated from petroleum contaminated soil proved to be the effective organisms for hydrocarbon degradation (Chaillan *et al.*, 2004). The degradation of polyaromatic hydrocarbons by *Sphingomonas* was reported by Daugulis and McCracken, (2003).

Fungal genera, including *Amorphoteca*, *Neosartorya*, *Talaromyces*, and *Graphium* and yeast genera, including, *Candida*, *Yarrowia*, and *Pichia* were isolated from petroleum contaminated soil and proved to be the potential organisms for degrading hydrocarbons (Chaillan *et al.*, 2004). Singh (2006), also discovered a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium*, and *Penicillium* which were also found to be the effective degrader of crude oil hydrocarbons. The yeast species, namely, *Candida lipolytica*, *Rhodotorula mucilaginosa*, *Geotrichum* sp. *and Trichosporon mucoides* isolated from contaminated water were noted to degrade petroleum compounds (Boguslawska-Was and Dabrowski, 2001).

Although algae and protozoa are the important members of the microbial community in both aquatic and terrestrial ecosystems, there are rare reports on their involvement in hydrocarbon biodegradation. Cerniglia *et al.* (1980), observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Protozoa, on the order hand, had not been proven to utilize hydrocarbons.

2.5. Enzymes participating in degradation of hydrocarbons

Van Beilen and Funhoff (2007), reported that cytochrome P450 alkane hydroxylases constitute a super family of ubiquitous Heme-thiolate Monooxygenases which play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation. Zimmer *et al.* (1996), stated that higher eukaryotes generally contain several different P450 families that consist of large number of individual P450 forms that may contribute as an ensemble of isoforms to the metabolic conversion of given substrate. In microorganisms such P450 multiplicity can only be found in few species.

Cytochrome P450 enzyme systems was found to be involved in biodegradation of petroleum hydrocarbons. The capability of several yeast species to use n-alkanes and other aliphatic hydrocarbons as a sole source of carbon and energy is mediated by the existence of multiple microsomal Cytochrome P450 forms. These cytochrome P450 enzymes had been isolated from yeast species such as *Candidamaltosa*, *Candida tropicalis*, and *Candida apicola* (Scheuer *et al.*, 1998). The diversity of alkaneoxygenase systems in prokaryotes and eukaryotes that are actively participating in the degradation of alkanes under aerobic conditions like Cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (e.g., *alkB*), soluble di-iron methane monooxygenases, and membrane-bound copper containing methane monooxygenases have been discussed by Van Beilen and Funhoff (2005).

2.6. Biosurfactants in hydrocarbon degradation

2.6.1. Biosurfactants

Ilori *et al.* (2005), Muthusamy *et al.* (2008) and Mahmound *et al.* (2008), defined biosurfactants as heterogeneous group of surface active chemical compounds produced by a

wide variety of microorganisms. According to Bai *et al.* (1997), surfactants enhance solubilisation and removal of contaminants. Barkay *et al.* (1999), reported that biodegradation is also enhanced by surfactants due to increased bioavailability of pollutants.

Bioremediation of oil sludge using biosurfactants was once been reported by Cameotra and Singh (2008). Microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate *Rhodococcus erythropolis* from soil contaminated with oily sludge was used in their study. The consortium was reported to be able to degrade 90% of hydrocarbons in 6 weeks in liquid culture. The ability of this consortium to degrade sludge hydrocarbons was tested in two separate field trials. In addition, the effect of two additives (a nutrient mixture and a crude biosurfactant preparation on the efficiency of the process was also assessed. The biosurfactant used was produced by a consortium member and was identified as being a mixture of 11 rhamnolipid congeners. The consortium degraded 91% of the hydrocarbon content of soil contaminated with 1% (v/v) crude oil sludge in 5 weeks. Separate use of any of the additive along with the consortium resulted into about 91–95% depletion of the hydrocarbon content in 4 weeks, with the crude biosurfactant preparation being a more effective enhancer of degradation. However, more than 98% hydrocarbon depletion was obtained when both additives were added together with the consortium. The data substantiated the use of a crude biosurfactant for hydrocarbon remediation.

According to Rahman *et al.* (2003), Cameotra and Singh (2008), *Pseudomonads* are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants. Among *Pseudomonads*, *Pseudomonas aeruginosa* is widely studied for the production of glycolipid type biosurfactants. However, glycolipid type biosurfactants are also reported from some other species like *Pseudomonas putida* and *Pseudomonas chlororaphis*. Nikolopoulou and Kalogerakis (2009), reported that biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize it. They also

stated that biosurfactants can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded.

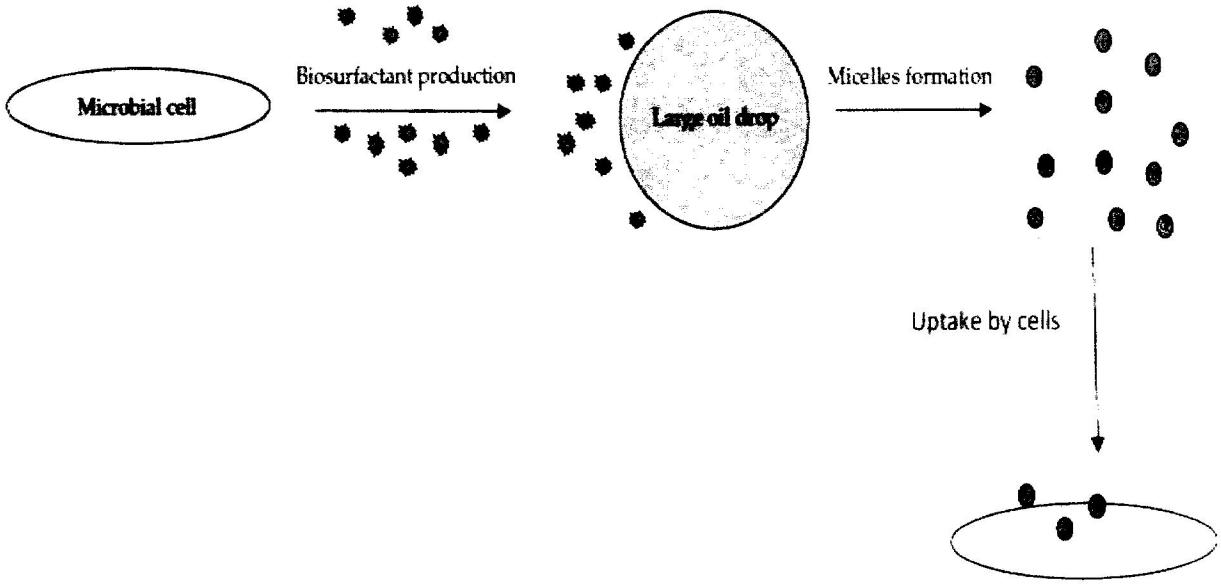


Figure 2: Mechanism of Uptake of Hydrocarbons by Biosurfactants (Source: Shukla and Cameotra, 2012)

2.6.2. Classification of biosurfactants

Biosurfactants are commonly classified based on their biochemical nature or the microbial producer species. Based on the structure, these compounds are classified into five main groups (Rahman and Gakpe, 2008; Daverey and Pakshirajan, 2009).

- Glycolipids: The degree of polarity depends on the hydrocarbons used as substrate; examples include rhamnolipids produced by *Pseudomonas aeruginosa* and sophorolipids produced by species of *Candida.*

- Lipopolysaccharides: These normally have a high molecular mass and are soluble in water; example is emulsan, an extracellular emulsifier produced from hydrocarbons by the bacteria *Acinotobacter calcoaceticus.*
- Lipopeptides: example: surfactin produced by *Bacillus subtilis* (one of the most potent biosurfactants reported in the literature).
- Phospholipids: They are structures common to many microorganisms; example is biosurfactant from *Corynebacterium lepus.*
- Fatty acids, neutral lipids (some classified as glycolipids) and hydrophobic proteins.

2.6.3. Uses of biosurfactants

The potential use of biosurfactants and biosurfactant producing microorganisms in bioremediation has been reported by numerous authors as indicated in the review made by Pacwa-Plociniczak *et al.* (2011).

Bordoloi and Konwar (2009), studied different strains of *Pseudomonas aeruginosa* and are found to be capable of degrading different types of polycyclic aromatic hydrocarbons such as pryene, fluorene and phenanthrene. Martins *et al.* (2009), evaluated the in situ bioremediation of a diesel oil spill, comparing the efficiency of biosurfactants to chemical remediation. The results reported that biosurfactants are efficient in the biodegradation of aliphatic and aromatic hydrocarbons up to 3 rings. Lin *et al.* (2010), compare different land farming methods widely used for petroleum hydrocarbon contaminated soil bioremediation. They found that the bioavailability of hydrocarbon was the limiting factor in the beginning of the degradation process. Therefore, bioaugmentation and biosurfactant addition showed the best result. Bioaugmentation involves the introduction of microbial strains to a contaminated site. Darvishi *et al.* (2011), studied the biosurfactant production potential of a microbial consortium of *Enterobacter cloacae and Pseudomonas sp.* isolated from a heavy crude oil contaminated site. They found that the consortium produces biosurfactants with heavy crude

oil as a soil carbon source. Whang *et al.* (2008), investigated the potential application of two biosurfactants for enhanced biodegradation of diesel-contaminated water and soil with bench-scale experiments. They focused on surfactin produced by *Bacillus subtilis* and rhamnolipid produced by *Pseudomonas aeruginosa*. They found that both biosurfactants are able to increase diesel solubility, and correlate positively on efficiency and rate of diesel biodegradation.

2.7. Types of bioremediation

Bioremediation as a treatment technique can be used in-situ or ex site.

A. **In-situ remediation:** are defined as those that are applied to soil at the site with minimal disturbance. This means that contaminants are treated without excavation or removal and transport from the site. Gruiz and Kriston (1995), reported that in-situ bioremediation have relatively low cost, little change in the soil structure, and result that may meet regulatory clean up guidelines. Fulekar (2009), reported that in-situ techniques leads to fewer disturbances, since they provide the treatment in place avoiding excavation and transport of contaminants. In-situ remediation therefore is a possible method when it is too expensive to excavate and transport the contaminated soil from the site.

B. **Ex situ remediation:** methods imply the excavation of the contaminated soils and the construction of a lined biotreatment cell on site. Advantages of ex situ treatment allows a better control of remediation parameters such as temperature, moisture content, nutrient concentration, and oxygen availability. The disadvantage is that excavation of the contaminated soil increases the cost of the operation (Fulekar, 2009).

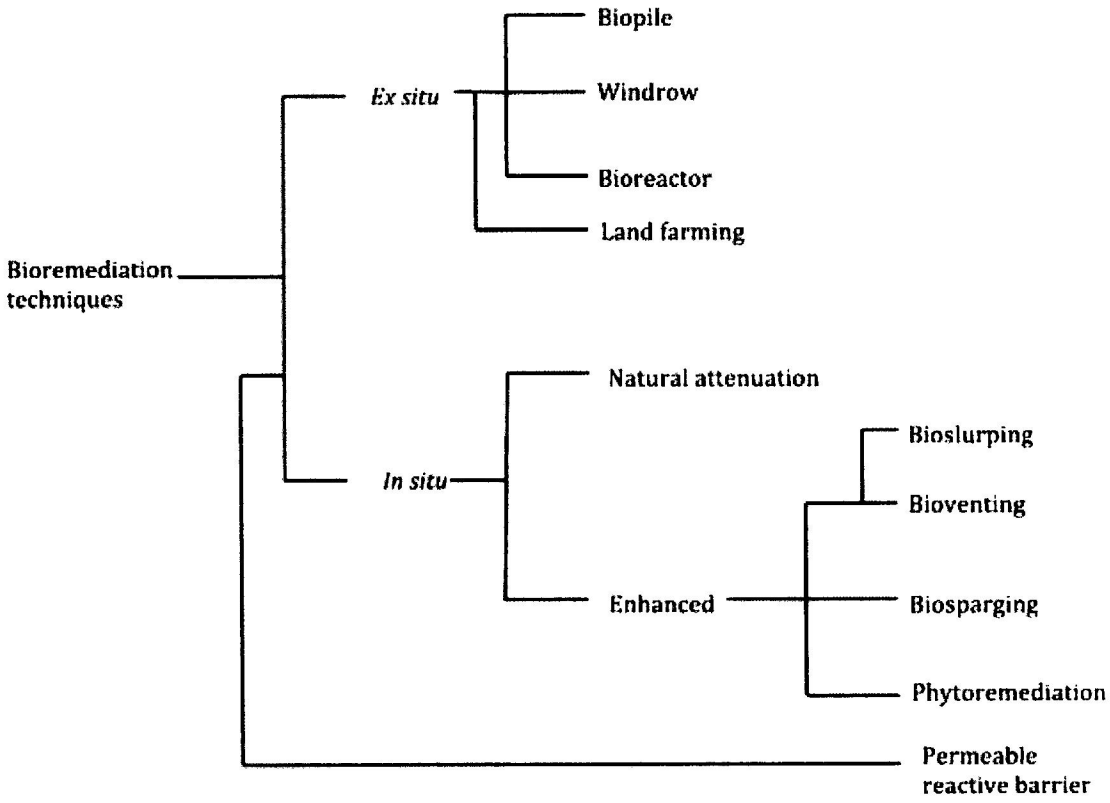


Figure 3: Chart for bioremediation techniques (Source: Christopher *et al.*, 2016)

2.8. Bioremediation techniques

Biopiles

Biopile bioremediation involves piling of above ground excavated polluted soil, followed by nutrient amendment, and sometimes aeration in order to enhance bioremediation by basically increasing microbial activities (Whelan *et al.*, 2015). The components of this technique include; aeration, irrigation, nutrient and leachate collection systems, and a treatment bed. The use of this particular ex situ technique is increasingly being considered due to its constructive features including cost effectiveness, which enables effective biodegradation on the condition that nutrient, temperature and aeration are adequately controlled (Whelan *et al.*, 2015). The application of biopile to polluted sites can help limit volatilization of low molecular weight (LMW) pollutants; it can also be used effectively to remediate polluted extreme environments such as the very cold regions (Dias *et al.*, 2015; Gomez and Sartaj, 2014).

Windrows

This is an ex situ bioremediation techniques which rely on periodic turning of piled polluted soil to enhance bioremediation by increasing degradation activities of indigenous and/or transient hydrocarbonoclastic bacteria present in polluted soil (Barr, 2002). The periodic turning of polluted soil, together with addition of water leads to aeration, uniform distribution of pollutants, nutrients and microbial degradative activities, thereby speeding up the rate of bioremediation, which can be accomplished through assimilation, biotransformation and mineralization (Barr, 2002). Windrow treatment when compared to biopile treatment, showed higher rate of hydrocarbon removal; however, the higher efficiency of the windrow towards hydrocarbon removal was as a result of the soil type, which was reported to be more friable (Coulon *et al.*, 2010).

Biostimulation

Some microorganisms are present in the contaminated site, but for effective remediation, growth of microorganism should be stimulated. Biostimulation is the process of adding nutrient, phosphorus, nitrogen, electron acceptor to stimulate existing indigenous microorganism in the soil (Vidali, 2001). This is the process of optimizing the environmental condition of the remediation site. Additives are usually added to the subsurface through injection wells. Subsurface characteristics such as groundwater velocity, hydraulic conductivity of the subsurface, and lithology of the subsurface are important in developing a biostimulation system (Vidali, 2001). The indigenous microorganism present in the soil is responsible for degradation of the pollutant, but biostimulation can be improved by bioaugmentation (Vidali, 2001).

Land farming

Land farming is a method in which contaminated soil is spread over a prepared bed along with some fertilizers and occasionally rotated (Spormann, 2000). It can also be defined as the technology that usually involves spreading excavated contaminated soils in a thin layer on the ground surface and stimulating aerobic microbial activity within the soils through aeration and/or the addition of minerals, nutrients, and moisture. It stimulates the activity of bacteria and enhances the degradation of oil (Spormann, 2000). It is also known as land treatment or land application is an above-ground remediation technology for soils that reduces concentrations of organic pollutants through biodegradation. As contaminated soil is treated in thin layers of up to 0.4 m thickness, it requires a large treatment area. To promote degradation enhancement of oxygen supply as well as mixing are done by ploughing, harrowing or milling at regular intervals. The treatment process is cost effective and can be adopted if sufficient land is available (Mohapatra, 2008).

Bioventing

This technique involves controlled stimulation of airflow by delivering oxygen to unsaturated (vadose) zone so as to increase bioremediation, by increasing activities of indigenous microorganisms (Mohapatra, 2008). In bioventing, amendments are made by adding nutrients and moisture to enhance bioremediation in order to achieve microbial transformation of pollutants to a harmless state (Philp and Atlas, 2005). This technique has gained popularity among other in situ bioremediation techniques especially in restoring sites polluted with light spilled petroleum products (Ho¨hener and Ponsin, 2014).

A study by Sui and Li (2011), modelled the effect of air injection rate on volatilization, biodegradation and biotransformation of toluene-contaminated site by bioventing. It was observed that at two different air injection rates (81.504 and 407.52 m3/d), no significant

difference in contaminant (toluene) removal was observed at the end of the study period (200 days). However, at the earlier stage of the study (day 100), it was observed that high air injection rate resulted in enhanced toluene removal by volatilization compared to low air injection rate. In other words, high air flow rate does not bring about increase in biodegradation rate nor make pollutant biotransformation more effective. This is due to early saturation of air (by high or low air injection rate) in the subsurface for oxygen demand during biodegradation. Nonetheless, low air injection rate resulted in a significant increase in biodegradation. It thus demonstrates that in bioventing, air injection rate is among the basic parameters for pollutant dispersal, redistribution and surface loss.

Biosparing

This technique is similar to bioventing in that air is injected into soil subsurface to stimulate microbial activities so as to promote pollutant removal from polluted sites. However, unlike bioventing, air is injected at the saturated zone, which can cause upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation. The effectiveness of biosparging depends on two major factors namely: soil permeability, which determines pollutant bioavailability to microorganisms, and pollutant biodegradability (Philp and Atlas, 2005). As with bioventing and soil vapour extraction (SVE), biosparing is similar in operation with a closely related technique known as in situ air sparging (IAS), which relies on high airflow rates to achieve pollutant volatilization, whereas biosparging promotes biodegradation.

Similarly, both mechanisms of pollutant removal are not mutually exclusive for both techniques. Biosparging has been widely used in treating aquifers contaminated with petroleum products, especially diesel and kerosene. Kao *et al.* (2008), reported that biosparging of benzene, toluene, ethylbenzene and xylene (BTEX)-contaminated aquifer plume resulted in a shift from anaerobic to aerobic conditions; this was evidenced by increased dissolved oxygen,

redox potentials, nitrate, sulphate and total culturable heterotrophs with a corresponding decrease in dissolved ferrous iron, sulphide, methane and total anaerobes and methanogens. The overall decrease in BTEX reduction (70 %) further indicates that biosparging can be used to remediate BTEX contaminated ground water. The major limitation however, is predicting the direction of airflow.

Bioaugmentation

Bioaugmentation is the addition, inoculation or introduction of a group of indigenous microbial strains or genetically engineered microbes to treat the contaminated soil or facilitate biodegradation. It is effective where native microorganisms are not identified in the soil or do not have the metabolic capability to perform the remediation process. Two factors limit the use of added microbial cultures in a land treatment unit:

a) Non-indigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and;
b) Most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed (Vidali, 2001).

Bioreactors

In this process contaminated soil are treated either in solid or slurry phase. The principle of solid phase reactors is mechanical decomposition of the soil by attrition and by intensive mixture of the components in a closed container. This ensures that the contaminants, microorganisms, nutrients, water and air are brought into permanent contact. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system (Jain *et al.*, 2011). A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid,

and gas) mixing condition to increase the bioremediation rate of soil-bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than in situ or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pre-treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor (Vidali, 2001).

2.9. Factors affecting biodegradation of petroleum hydrocarbon

2.9.1. Temperature

Jain *et al.* (2011), stated that temperature plays very important role in biodegradation of petroleum hydrocarbon, firstly by its direct effect on the chemistry of the pollutants and secondly its effect on the physiology and diversity of the microorganism. Tang *et al.* (2012), reported that the rate of biodegradation generally decreases with the decreasing temperature. He further stated that a high temperature induces a high rate of biological degradation processes in the soil. The rate of degradation can double for every 10°C rise in temperature. Highest degradation rates occur generally in the range 30-40°C. Venosa and Zhu (2003), reported that the ambient temperature of an environment affects both the property of the spilled oil and the activity of microorganism. It was also reported by Fought *et al.* (1996), that at low temperature the viscosity of oil increases, while the volatility of toxic low molecular weight hydrocarbons is reduced, delaying the degradation. Thus, temperature affects solubility of hydrocarbons.

2.9.2. Nutrient

Nutrient is an important parameter in biodegradation. Choi *et al.* (2002), observed that the nutrients status of soil has direct impacts on microbial activity and biodegradation. Addition of nutrients is necessary to enhance the biodegradation of oil pollution. The effectiveness of fertilizers for the crude oil bioremediation in subarctic intertidal sediments was studied by Pelletier *et al.* (2004). Use of poultry manure as organic fertilizer in contaminated soil was also reported by Okolo *et al.* (2005), and biodegradation was found to be enhanced in the presence of poultry manure alone. Maki *et al.* (2005), reported that photo-oxidation increased the biodegradability of petroleum hydrocarbon by increasing its bioavailability and thus enhancing microbial activities. It was reported by Chaillan *et al.* (2006), that excessive nutrient concentration can inhibit biodegradation activity.

2.9.3. Oxygen

Oxygen is another parameter that affects biodegradation. Oxygen determines the bacterial pattern of dissimilatory and energy yielding process. Fulekar (2009), reported that catabolism of aliphatic, cyclic and aromatic hydrocarbon by bacteria and fungi in the initial step involves the oxidation of substrate by oxygenases, for which molecular oxygen is required. Oxygen available in soil depends on the rate of O_2 consumption by microorganism, the type of soil and the presence of utilizable substrates which lead to oxygen depletion. In hydrocarbon aerobic bioremediation, oxygen availability is a critical factor. Bacteria activity proceeds more rapidly if sufficient oxygen is provided. During aerobic biodegradation, molecular oxygen is reduced to water while petroleum hydrocarbon is oxidized to create energy, cell mass, and carbondioxide (Dineen *et al.*, 1990).

2.9.4. Moisture

Microorganisms require water for microbial growth and for diffusion of nutrients and by-products during the degradation process. According to Jain *et al.* (2011), if the soil is too dry, many rnicroorganisms will die. If water content of the soil is too high, oxygen transfer to microorganisms will be resisted by the flooded soil and the rate of the hydrocarbon degradation will be reduced. The optimum soil water content for bioremediation is dependent on the soil type. Baker and Herson (1994a), reported that the optimum activity occurs when the soil moisture is 50-80% of the field capacity, also termed the water holding capacity which is defined as "the amount of the water remaining within the soil after gravitational water has drained away or the percentage of water in a soil when it was saturated". When moisture content is lower than 10% of the holding capacity, the bioactivity becomes marginal (Testa and Winegardner, 1991).

2.9.5. pH

Biological activity in the soil can be affected by the pH. Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11 in alkaline deserts (Tang *et al.*, 2012). Some microorganisms can survive in a wide range of pH, but others are sensitive to small variations. The bacteria grow better in pH values between 6.5 and 8.5 but fungi is more acid tolerant (Tang *et al.*, 2012). Therefore, extreme pH of soil would have a negative influence on the ability of microbial populations to degrade hydrocarbons. Bioremediation is therefore favoured by near neutral pH values (6-8). Neutral pH is favourable by most heterotrophic bacteria and fungi. Soil pH can be adjusted if necessary to enhance microbial activity (Jain *et al.*, 2011).

2.10. Advantages and pitfalls of bioremediation

2.10.1. Advantages of bioremediation

Bioremediation is an important tool in the petroleum hydrocarbon contaminated environment compared to conventional method due to the following reasons (Roldán *et al.*, 2007, Gruiz and Kriston, 1995):

- Bioremediation is an environment friendly approach and is therefore accepted by public as a remedy in the treatment of contaminants.
- Bioremediation is less expensive as compared with other physiochemical techniques (land filling and incineration).
- Bioremediation can be done on site and site disruption is minimal.
- It eliminate waste and also eliminate the chance of future liability associated with treatment and disposal of contaminated material.
- The microorganisms involved in the degradation of contaminant increases in their number till the contaminant is present. After the degradation of contaminant the microbial population itself decreases naturally.
- Bioremediation transforms the toxic substrates to harmless products such as CO_2 (utilized by plants in photosynthesis), H_2O and fatty acids.

2.10.2. Pitfalls of bioremediation

According to Vidali (2001), the disadvantages include:

- Only biodegradable compounds are capable of undergoing bioremediation. Not every compound is capable of fully degrading quickly.

- The product of biodegradation may potentially be even more persistent or toxic than the original contaminant.
- Biological functions are usually extremely specific and require the presence of microbes that are capable of metabolizing the contaminants. In order for the correct microbes to be present, the appropriate environmental conditions, levels of nutrients, and contaminants need to be met.
- Compared to other treatment technologies, bioremediation often takes more time.
- Scaling up the size of studies from small initial studies to commercial-scale field operations is difficult.

CHAPTER THREE

3.0. MATERIALS AND METHODOLOGY

3.1. MATERIALS

Agar: Nutrient agar (NA), Nutrient broth, Bushnell Haas (BH) mineral salt medium, urea agar, citrate agar, TSI agar, MRVP agar, peptone water.

Reagents: Used engine oil, distilled water, 3% hydrogen peroxide (H_2O_2), aluminium chloride, aluminium nitrate, aluminium sulphate, 70% ethanol, alpha-napthol, potassium hydroxide, bromothymol, sugars (fructose, sucrose, glucose, Mannitol), methyl red reagent, malachite green, immersion oil, fucin, lugol's iodine, crystal violet, acetone, safranin, Kovac's reagent, oxidase reagent, kerosene, diesel.

Equipment: Microscope, incubator, shaker incubator, oven, pH meter, autoclave, Durham tube, polythene, sterile spatula, weighing balance, vortex mixer, test tubes, beaker, petri dishes, conical flask, bijou bottle, cover slip, slide, syringe, inoculating loop, inoculating wire, Bunsen burner.

3.2. METHODOLOGY

3.2.1. Study site

Study site selected were different mechanic workshops in Oye-Ekiti and Ilupeju-Ekiti. Site were heavily contaminated with spent engine oil.

3.2.2. Sampling

Soils contaminated with used engine oil were collected from four different mechanic workshop. They were designated as B, C, D and E. The selected site was within Oye-Ekiti and Ilupeju-Ekiti. At each identified mechanic workshop, two samples were collected from

different contaminated spot in the workshop. A total of 8 samples were aseptically taken using a sterilized spatula into sterile nylon bags. Soil from each sampling site was transferred into a sterilized polythene bag and was taken to the laboratory for analysis and was later stored at 4^0C. Soil samples were taken many meters away from the contaminated site on each mechanic workshop site, which was studied as negative control. (David *et al.*, 2016).

3.2.3. Isolation of microorganism

Each of the samples were prepared by serial dilution and pour plate technique. Nutrient Agar (NA) medium was used for the culturing of bacteria, the media was prepared according to the manufacturers specifications. About 10g of soil sample from each source was suspended in 90ml of distilled water and vortexed. Tenfold serial dilution was done. About 0.1ml of the 10^{-3} and 10^{-4} diluent was inoculated on nutrient agar in duplicate and incubated at 37^0C for 24 hrs (Ravi and Praveen, 2016).

3.2.4. Isolation of oil-degrading bacteria

Sterilized Bushnell Haas (BH) mineral salt agar [(g/l): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), K_2HPO_4 (1.0), NH_3NO_2 (1.0), $FeCl_3$ (0.05), Agar agar (20) and pH was adjusted to 7.0 ± 0.2] supplemented with 1% used engine oil was used to isolate the bacteria. The used engine oil serves as carbon source (Sadaf *et al.*. 2017). About 0.5g of fucin per 100ml was added to the medium to prevent the fungal growth. Tenfold serial dilution was done and about 0.2ml of the 10^{-2} and 10^{-3} diluent was inoculated on Bushnell Haas Agar in duplicate and was incubated at 37^0C for 7 days (Jyothi *et al.*, 2012).

3.3. Biochemical characterization

Each bacterial isolate was examined microscopically through gram staining. Biochemical tests was performed to identify the isolates to the genus level. The biochemical tests that were carried out include motility test, spore staining, catalase test, urease test, citrate

utilization test, oxidase test, indole test, methyl red test, Voges Proskaeur, Triple sugar iron test and sugar (glucose, sucrose, fructose) fermentation (Cheesbrough, 2006).

3.3.1. Gram Stain

A thin smear of the test organism was made on a clean grease-free slide. It was allowed to air-dry and then heat-fixed by passing it over a flame thrice. The slide was carefully placed on a staining rack and flooded with crystal violet solution and left for 60 seconds before being washed off with clean water. Lugol's iodine solution was added and left for 60 seconds and washed off with clean water. The slide was decolorized with acetone and was rinsed with clean water immediately and was counterstained with safranin. It was allowed to stand for 2 minutes, and was washed off with clean water and was air dried. The slide was thereafter examined under the oil immersion objective lens of the microscope.

3.3.2. Catalase Test

A drop of 3% hydrogen peroxide solution was introduced on a microscope slide and a loopful of the test organism was added. The appearance of sustained bubbles mean a positive reaction while the absence of bubbles mean a negative result.

3.3.3. Indole Test

5ml of peptone water was transferred into a test tube and autoclaved at 121°C for 15 minutes and was allowed to cool. The test isolate was thereafter inoculated into the broth and was incubated at 37°C for 24 hours. 3 drops of Kovac's reagent was added to the medium. A pink to red coloration mean a positive result while the presence of yellow colour mean a negative result.

3.3.4. Oxidase Test

A piece of filter paper was soaked with some drops of oxidase reagent (tetramethyl phenylenediamine dihydrochloride). The test organism was then smeared on the soaked filter paper. A change to deep purple colour within 10 seconds mean a positive reaction while no change in colour mean negative reaction.

3.3.5. Methyl Red (MR) Test

The test organism was introduced into MRVP broth that has already been prepared and sterilized and was incubated at 37^0C for 24 hours. 5 drops of methyl red reagent was added, mixed and the result was read. A red coloration mean a positive result while a yellow coloration mean a negative result.

3.3.6. Voges Proskaeur (VP) Test

The test organism was introduced into MRVP broth that has already been prepared and sterilized and was incubated at 37^0C for 24 hours. 5 drops of Baritt A (alpha-naphtol) and Baritt B (potassium hydroxide) reagents was added, mixed and the result was read. A pink burgundy coloration mean a positive result while a yellow coloration mean a negative result.

3.3.7. Citrate Utilization Test

Simmon citrate agar was prepared according to manufactures specification and was introduced into Bijou bottles and was autoclaved at 121^0C for 15 minutes. Upon cooling, the test organism was streaked on the agar and was incubated at 37^0C for 24 hours. A change in colour of the medium from green to blue mean a positive result while no colour change mean a negative result.

3.3.8. Urease Test

Urea agar was prepared according to the manufacturer's specification and was introduced into Bijou bottles and was autoclaved at 121^{0}C for 15 minutes. Upon cooling, the isolate was aseptically inoculated into the bottles which was then incubated at 37^{0}C for 24 hrs. A change of colour yellow to pink mean the production of urease which is a positive result while no colour change mean negative result.

3.3.9. Sugar Fermentation Test

Peptone water was prepared according to the manufacturer's instruction and methyl red indicator solution was added until there is change in colour. The test sugar (1%) was also added and the mixture was distributed into test tubes containing Durham tubes. The tubes was sterilized by autoclaving at 121^{0}C for 15 minutes and was allowed to cool. Upon cooling, the tubes was inoculated with the test organism and incubated at 37^{0}C for 48 hours.

3.3.10. Motility Test

Semi-solid nutrient agar was prepared, distributed in test tubes and was autoclaved and was then allowed to cool. The tubes were stab/inoculated with the test organism with a sterile straight wire and incubated at 37°C for 24 hours. The bacteria that grow along the straight line of the stab indicate non-motile ones while the motile bacteria grow away from the line of the stab.

3.3.11. Triple Sugar Iron (TSI)

Triple Sugar Iron agar was prepared, distributed in test tubes and was autoclaved and was slanted and was then allowed to cool. The tubes were inoculated with the test organism with a sterile inoculating loop and was incubated at 37°C for 24 hours. If both the butt and slant turn yellow, it mean that there is fermentation of lactose, glucose and sucrose; if only butt turn

yellow, it mean that there is fermentation of glucose; if only the slant colour turns yellow, it mean that there is fermentation of lactose and/or sucrose; if there is no colour change in both the butt and slant, it mean that there is no fermentation of glucose, sucrose & lactose; and black colouration shows H_2S production.

3.3.12. Spore staining

A heat fixed smear of the isolate was prepared on a grease free slide. The smear was stained with malachite green solution and was steamed for 5-10 minutes ensuring that the stain does not dry out. The stain was carefully rinsed out with clean water and counter stained with safranin solution for 15 seconds, it was then washed with water and was blotted dry and examined under oil immersion objective of a microscope, spores stained green while vegetative cells stained red.

3.4. Determination of growth rate

Nutrient broth was prepared and 10mls was dispensed into six test-tubes. It was then autoclaved at 121°C for 15mins. The six isolates were inoculated into each test-tube (one test-tube for each isolate) and was incubated for 48hrs. Bushnell Hass medium (broth) was prepared and 100ml was dispensed into seven conical flask and was sterilized using the autoclave at 121°C for 15mins. 1ml of the inoculated nutrient broth was dispensed into the sterilized Bushnell Hass medium (broth) and the seventh conical flask was not inoculated (which serve as the blank/control). They were incubated using the shaker incubator for 5 days (Mandri and Lin, 2007).

The growth rate was observed using two methods: Optical Density and Colony count.

The optical density was checked each day (day 0 to day 4) using the spectrophotometer with wave length 600nm. The number of colonies were counted by culturing on Nutrient agar each

day. Serial dilution was done and 0.1ml of diluent 4 (10^{-4}) was cultured using pour plate technique for each day and the total viable count was observed (Mandri and Lin, 2007).

3.5. Determination of the effect of different growth conditions on bacterial isolates

3.5.1. Effect of different carbon sources

Nutrient broth was prepared and 10mls was dispensed into two test-tubes. It was then autoclaved at 121°C for 15mins. The two isolates with the best growth rate (*Pseudomonas* sp. and *Micrococcus* sp.) were inoculated into each test-tube (one test-tube for each isolate) and was incubated for 48hrs. Bushnell Hass medium (broth) was prepared using three different carbon source (used engine oil, diesel and kerosene) and 100ml was dispensed into nine conical flask (3 conical flask for each carbon source) and was sterilized using the autoclave at 121°C for 15mins. 1ml of the inoculated nutrient broth was dispensed into the sterilized Bushnell Hass medium (broth) and the three of the nine conical flask was not inoculated (which serve as the blank control). They were incubated using the shaker incubator for 5 days.

The growth rate was observed using two methods: Optical Density and Colony count.

The optical density was checked each day (day 0 to day 4) using the spectrophotometer with wave length 600nm. The number of colonies were counted by culturing on Nutrient agar each day. Serial dilution was done and 0.1ml of diluent 4 (10^{-4}) was cultured using pour plate technique for each day and the total viable count was observed (Mandri and Lin, 2007).

3.5.2. Effect of different nitrogen sources

Nutrient broth was prepared and 10mls was dispensed into two test-tubes. It was then autoclaved at 121°C for 15mins. The two isolates with the best growth rate (*Pseudomonas* sp. and *Micrococcus* sp.) were inoculated into each test-tube (one test-tube for each isolate) and was incubated for 48hrs. Bushnell Hass medium (broth) was prepared using three different

nitrogen source (aluminium sulphate, aluminium nitrate, aluminium chloride) and 100ml was dispensed into nine conical flask (3 conical flask for each nitrogen source) and was sterilized using the autoclave at 121°C for 15mins. 1ml of the inoculated nutrient broth was dispensed into the sterilized Bushnell Hass medium (broth) and the three of the nine conical flask was not inoculated (which serve as the blank/control). They were incubated using the shaker incubator for 5 days.

The growth rate was observed using two methods: Optical Density and Colony count. The optical density was checked each day (day 0 to day 4) using the spectrophotometer with wave length 600nm. The number of colonies was counted by culturing on Nutrient agar each day. Serial dilution was done and 0.1ml of diluent 4 (10^{-4}) was cultured using pour plate technique for each day and the total viable count was observed (Mandri and Lin, 2007).

3.5.3. Effect of different pH

Nutrient broth was prepared and 10mls was dispensed into two test-tubes. It was then autoclaved at 121°C for 15mins. The two isolates with the best growth rate (*Pseudomonas* sp. and *Micrococcus* sp.) were inoculated into each test-tube (one test-tube for each isolate) and was incubated for 48hrs. Bushnell Hass medium (broth) was prepared using three different pH (6, 7, and 8) and 100ml was dispensed into nine conical flask (3 conical flask for each pH) and was sterilized using the autoclave at 121°C for 15mins. 1ml of the inoculated nutrient broth was dispensed into the sterilized Bushnell Hass medium (broth) and the three of the nine conical flask was not inoculated (which serve as the blank/control). They were incubated using the shaker incubator for 5 days.

The growth rate was observed using two methods: Optical Density and Colony count. The optical density was checked each day (day 0 to day 4) using the spectrophotometer with wave length 600nm. The number of colonies was counted by culturing on Nutrient agar each

day. Serial dilution was done and 0.1ml of diluent 4 (10^{-4}) was cultured using pour plate technique for each day and the total viable count was observed (Mandri and Lin, 2007).

CHAPTER FOUR

RESULT

4.1. Sampling

Soils samples contaminated with used engine oil were collected from four different mechanic workshop in Oye-Ekiti and Ilupeju-Ekiti, Ekiti State. The sampling site is shown in Plate 1. They were designated as B (with latitude 7°47'50.088''N, longitude 5°19'28.758''E, degree 22°NE), C (with latitude 7°47'56.970''N, longitude 5°19'32.844''E, degree 26°NE), D (with latitude 7°47'47.534''N, longitude 5°21'33.720''E, degree 28°NE) and E (with latitude 7°47'47.364''N, longitude 5°21'22.716''E, degree 23°NE).

4.2. Total Number of Heterotrophic Bacteria

The bacterial count at 24hours for dilution factors 3 and 4 were recorded as shown in Table 2. The result revealed that the colony count for each sample for diluent 3 & 4 as B ($2.6x10^{-1}$ cfu/ml and $1.2x10^{-2}$ cfu/ml), C ($6.0x10^{-2}$ cfu/ml and $4.0x10^{-3}$ cfu/ml), D ($3.0x10^{-1}$ cfu/ml and $1.3x10^{-2}$ cfu/ml) and E ($6.0x10^{-2}$ cfu/ml and $4.0x10^{-3}$ cfu/ml) respectively. Sample D had the highest bacterial count for both diluent 3 and 4 ($3.0x10^{-1}$ cfu/ml and $1.3x10^{-2}$ cfu/ml respectively) while sample E had the least bacterial count for both diluent 3 and 4 ($6.0x10^{-2}$ cfu/ml and $4.0x10^{-3}$ cfu/ml respectively) (Table 2).

4.3. Biochemical characterization of Bacterial Isolates

A total number of 36 isolates were isolated by plating on Bushnell Hass agar with the composition [(g/l): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), K_2HPO_4 (1.0), NH_3NO_2 (1.0), $FeCl_3$ (0.05), Agar agar (20) and pH was adjusted to 7.0 ± 0.2, 1% engine oil]. Each bacterial isolates were designated according to the site from which they were isolated. The isolates were designated as B1, B2, B3..., C1, C2, C3..., D1, D2, D3..., E1, E2, E3..., for site B, C, D and

E respectively. Each bacterial isolate were kept at 4°C in a refrigerator for further studies. Catalase test, oxidase test, citrate test, urease test, spore staining, MR test, VP test, indole test, sugar fermentation test, Triple sugar test and motility test was done. Based on the morphological & microscopic observation and biochemical tests all the bacteria isolates were identified till genus level (Table 3) using the Bergey's Manual of Determinative Bacteriology.

The result in Table 3 revealed that all the bacterial isolates were positive to catalase test and citrate test, all the bacterial isolates were negative to indole test. Only *Bacillus* sp. was positive for endospore staining and also only *Staphylococcus aureus* gave positive result to mannitol fermentation test. Only *Staphylococcus epidermidis* was positive to Voges proskaeur test. Only *Alcaligenes* sp. was negative to urease test. Only *Pseudomonas* sp. was negative to glucose, fructose and sucrose fermentation test and was positive to H_2S test (Table 3).

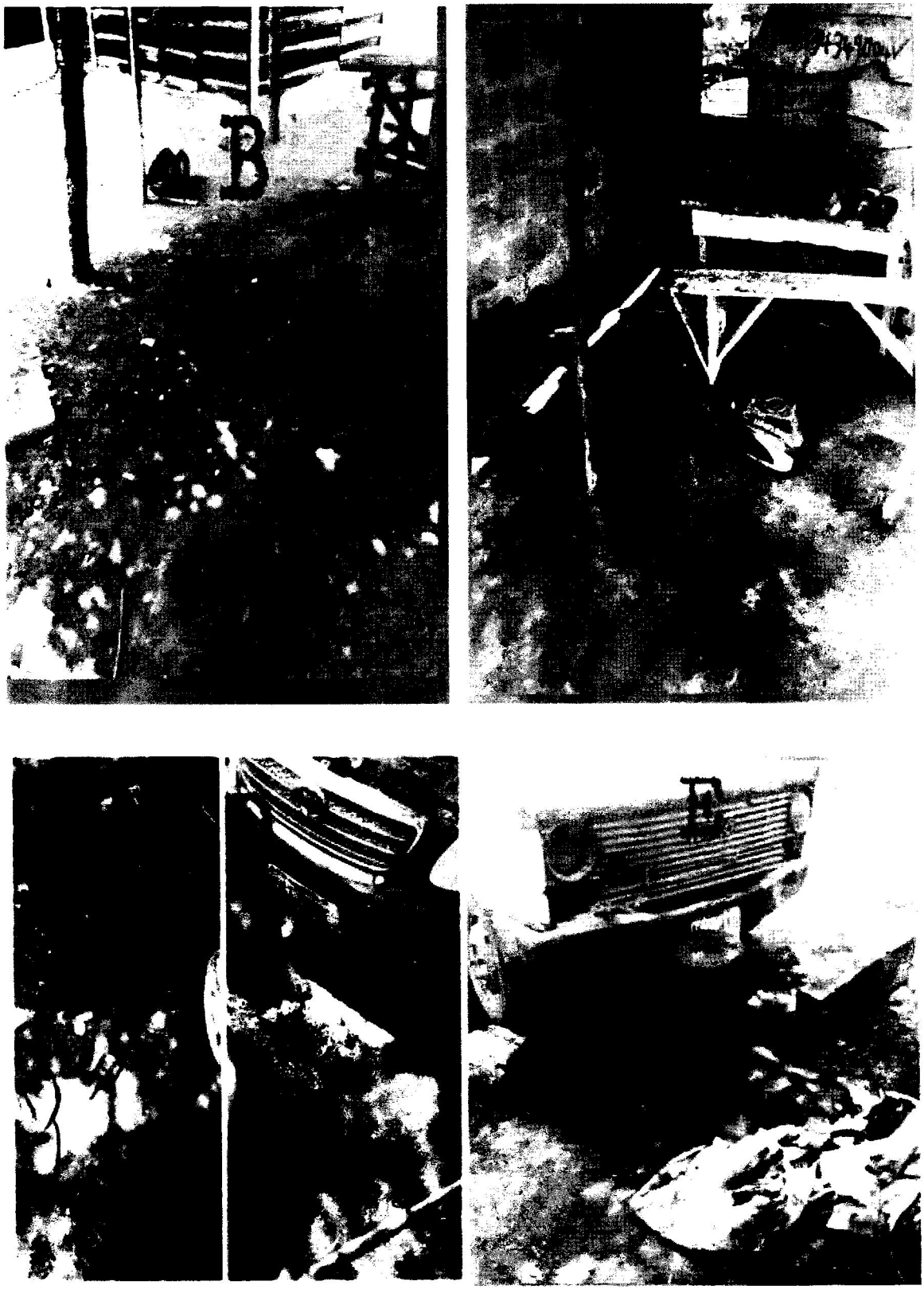


Plate 1: Pictures of the selected mechanic workshops

Table 2: Colony Count on Nutrient Agar at 24hours

Sites	Diluent 3	Diluent 4
B	$2.6x10^{-1}$	$1.2x10^{-2}$
C	$6.0x10^{-2}$	$4.0x10^{-3}$
D	$3.0x10^{-1}$	$1.3x10^{-2}$
E	$2.7x10^{-1}$	$2.0x10^{-2}$

ble 3: Cultural, Morphological and Biochemical Characteristics of Isolated Bacteria

ates	Cultural Characteristics						Morphological Characteristics		Biochemical Characteristics															
	Elevation	Margin	Form	Size	Texture	colour	Shape	Gram	Catalase	Oxidase	Indole	Citrate	Urease	Spore	MR	VP	H_2S	Motility	Mannitol	Glucose	Fructose	Sucrose	Probable Organism
1	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
2	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
3	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
4	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
5	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
6	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
1	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
2	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
3	Raised	Entire	Circular	Moderate	Moist	Green	Bacilli	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	P.S
4	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
5	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
6	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
1	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	S.E
2	Flat	Irregular	Circular	Big	Dry	White	Bacilli	+	+	+	-	+	+	+	+	-	-	+	-	+	+	+	B.S
3	Raised	Entire	Circular	Moderate	Moist	Cream	Bacilli	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	A.S
4	Raised	Entire	Circular	Moderate	Moist	Cream	Bacilli	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	A.S
5	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
6	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
7	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
8	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
9	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
10	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	S.E
11	Flat	Irregular	Circular	Big	Dry	White	Bacilli	+	+	+	-	+	+	+	+	-	-	+	-	+	+	+	B.S
12	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	S.E
13	Raised	Entire	Circular	Moderate	Moist	Yellow	Cocci	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+	M.S
1	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
2	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
3	Raised	Entire	Circular	Moderate	Moist	Green	Bacilli	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	P.S
4	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	S.E
5	Raised	Entire	Circular	Moderate	Moist	Green	Bacilli	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	P.S

6	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
7	Raised	Entire	Circular	Moderate	Moist	Cream	Bacilli	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	A.S
8	Raised	Entire	Circular	Moderate	Moist	Green	Bacilli	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	P.S
9	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	-	-	-	+	+	+	+	S.A
0	Raised	Entire	Circular	Small	Moist	White	Cocci	+	+	-	-	+	+	-	+	+	-	-	-	+	+	+	S.E
1	Raised	Entire	Circular	Moderate	Moist	Cream	Bacilli	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	A.S

ys:

Positive

Negative

A= *Staphylococcus aureus*

E= *Staphylococcus epidermidis*

S= *Micrococcus* sp.

S= *Alcaligenes* sp.

B= Mechanic workshop B

C= Mechanic workshop C

D= Mechanic workshop D

E= Mechanic workshop E

B.S= *Bacillus* sp.

P.S= *Pseudomonas* sp.

4.4. Determination of Growth Rate

Each bacterial isolate was inoculated into Bushnell Hass broth in order to know which organism among the six identified isolates can grow best in the environment. The bacterial growth rate was determined by measuring the optical density using the spectrophotometer at wavelength 600nm (Figure 4) and also by colony count (Figure 5). The result revealed that *Pseudomonas* sp. and *Micrococcus* sp. had the highest number of colonies and highest optical density starting from day 0 to day 4. For other organisms (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Alcaligenes* sp.) had increase in optical density and number of colonies from day 0 to day 2 but decreased from day 2 to day 4. *Bacillus* sp. had increase in optical density and number of colonies from day 0 to day 3 but decreased from day 3 to day 4. (Figure 4 & 5)

4.5. Determination of the Effect of Different Growth Conditions on Bacterial Isolates

4.5.1. Effect of different carbon sources

Different carbon sources were used to determine the best organism that can utilize the different carbon sources for their growth and metabolic activities. The different carbon sources used include; used engine oil, kerosene and diesel. The bacterial growth rate was determined by measuring the optical density using the spectrophotometer at wavelength 600nm (Figure 6) and also by colony count (Figure 7). The result revealed that *Pseudomonas* sp. was able to utilize used engine oil and kerosene as its carbon source compared to diesel. For used engine oil and kerosene there was increase in the optical density and number of colonies from day 0 to day 4 but for diesel there was decrease from day 2. For engine oil the OD and number of colonies increases from -0.043A to 0.128A and 180-280 respectively. For kerosene the OD and number of colonies increases from -0.01A to 0.131A and 196-310 respectively (Figure 6 & 7). *Micrococcus* sp. was able to utilize used engine oil as its carbon source compared to diesel and

kerosene. For used engine oil there was increase in the optical density and number of colonies from day 0 to day 4 but for diesel there was decrease from day 2 and for kerosene there was decrease from day 1 (Figure 6 & 7). For engine oil the OD and number of colonies increases from -0.049A to 0.327A and 190-320 respectively. The best carbon source for the two organisms was used engine but *Pseudomonas* sp. was also able to utilize kerosene. *Micrococcus* sp. was able to utilize used engine oil more than *Pseudomonas* sp. (Figure 6 & 7)

4.5.2. Effect of Different Nitrogen Sources

Different nitrogen sources were used in order to know the best nitrogen source that will enhance the growth of the isolates. The different nitrogen sources used include; ammonium nitrate, ammonium sulphate and ammonium chloride. The result revealed that both *Pseudomonas* sp. and *Micrococcus* sp. was able to utilize ammonium nitrate as the best nitrogen source compared to ammonium sulphate and ammonium chloride. For ammonium nitrate there was increase in the optical density (Figure 8) and number of colonies (Figure 9) from day 0 to day 4. (For *Pseudomonas* sp. the OD and number of colonies increases from 0.161A to 0.225A and 180-320 respectively; while for *Micrococcus* sp. the OD and number of colonies increases from 0.158A to 0.239A and 185-330 respectively). For both organisms for ammonium chloride there was decrease in optical density from day 3. *Micrococcus* sp. was able to utilize ammonium sulphate more than *Pseudomonas* sp. (Figure 8& 9).

4.5.3. Effect of Different pH

Different pH were used in order to know the pH that the isolates can grow best. The different pH used are 6, 7, and 8. The result revealed that both *Pseudomonas* sp. and *Micrococcus* sp. was able to grow best at pH 7and 8 compared to pH 6. For pH 7 and 8 there was increase in the optical density (Figure 10) and number of colonies (Figure 11) from day 0 to day 4, (At pH 7 for *Pseudomonas* sp. the OD and number of colonies increases from -0.040A

to 0.180A and 160-320 respectively; while for *Micrococcus* sp. the OD and number of colonies increases from -0.075A to 0.127A and 140-305 respectively. At pH 8 for *Pseudomonas* sp. the OD and number of colonies increases from 0.025A to 0.121A and 120-297 respectively; while for *Micrococcus* sp. the OD and number of colonies increases from 0.045A to 0.113A and 150-290 respectively). For both organisms for pH 6 there was decrease in optical density and number of colonies from day 3 (Figure 10&11).

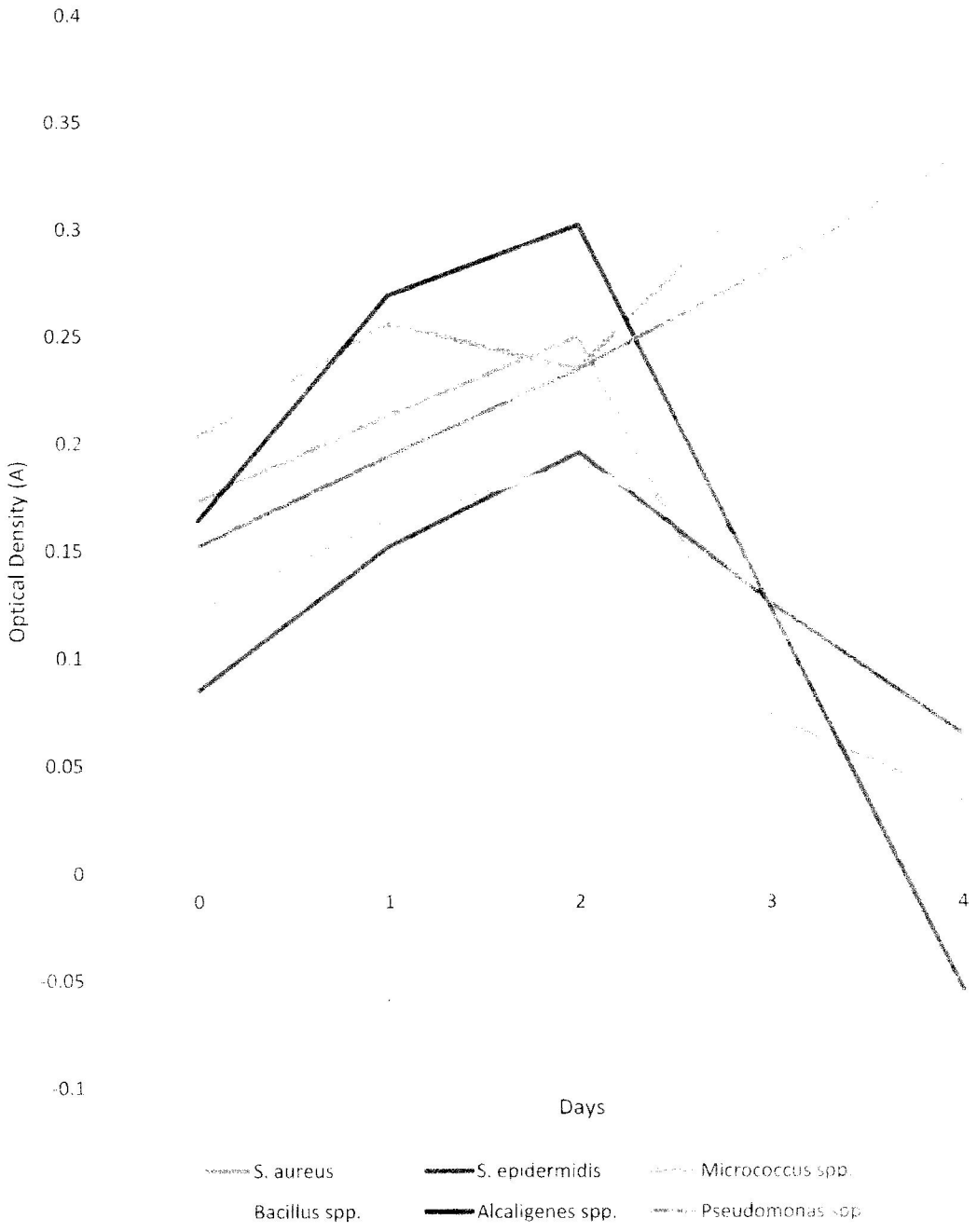


Figure 4: Optical Density of each bacteria isolates from Day 0-4

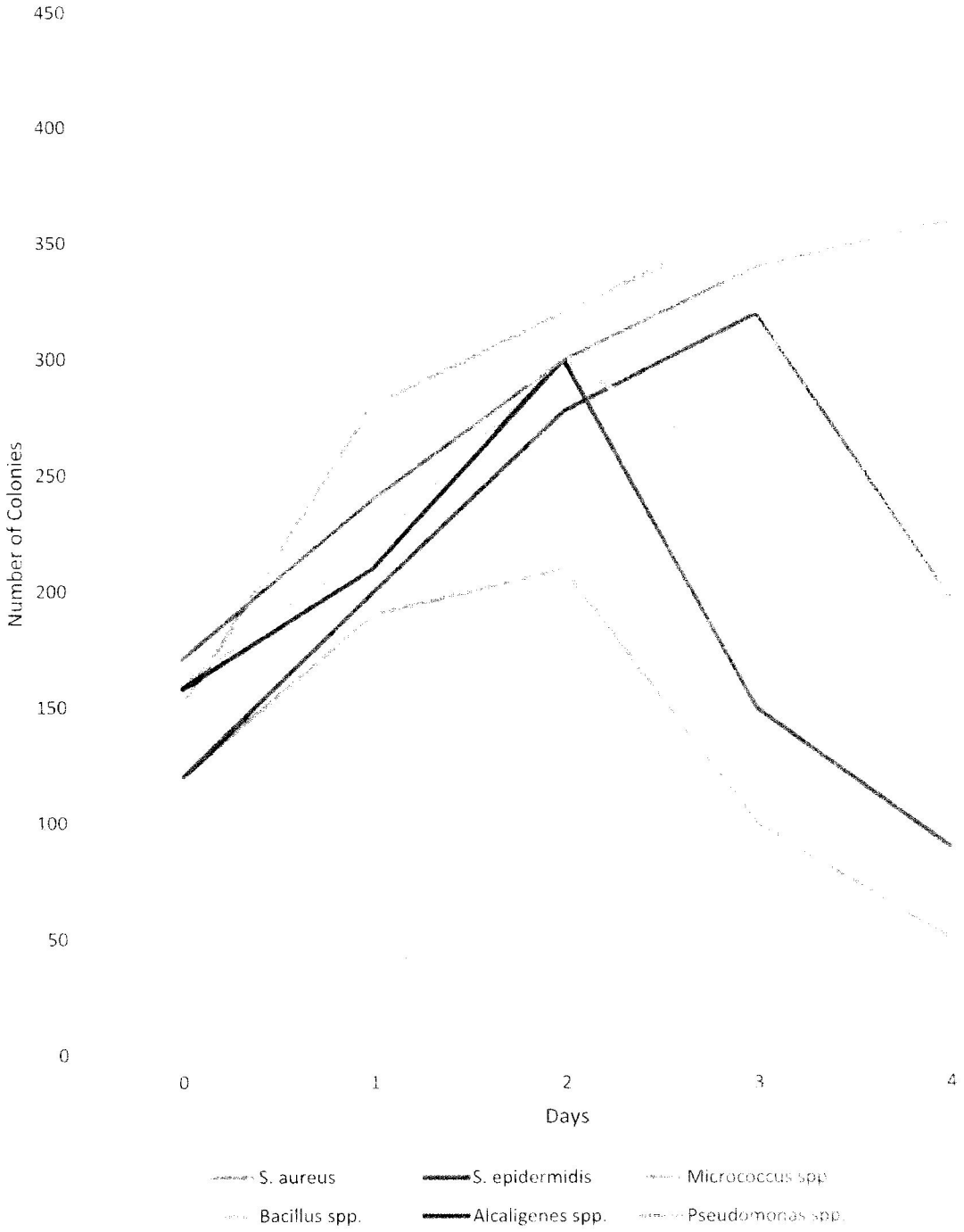


Figure 5: Total viable count of each bacteria isolates from Day 0-4

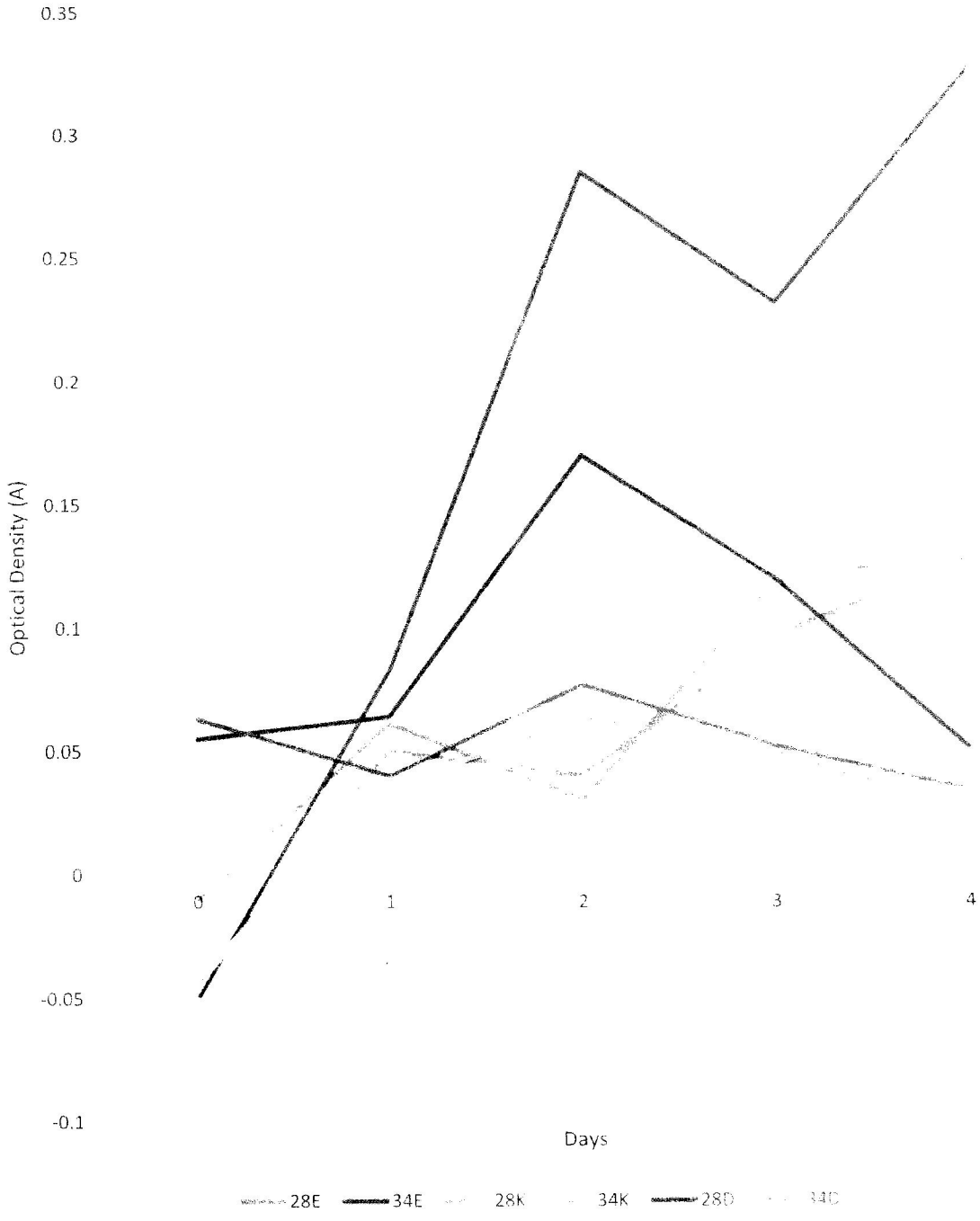


Figure 6: Optical density of bacteria subjected to different carbon sources **Keys:**

E= Engine oil, K= Kerosene, D= Diesel, 28= *Pseudomonas* sp., 34= *Micrococcus* sp.

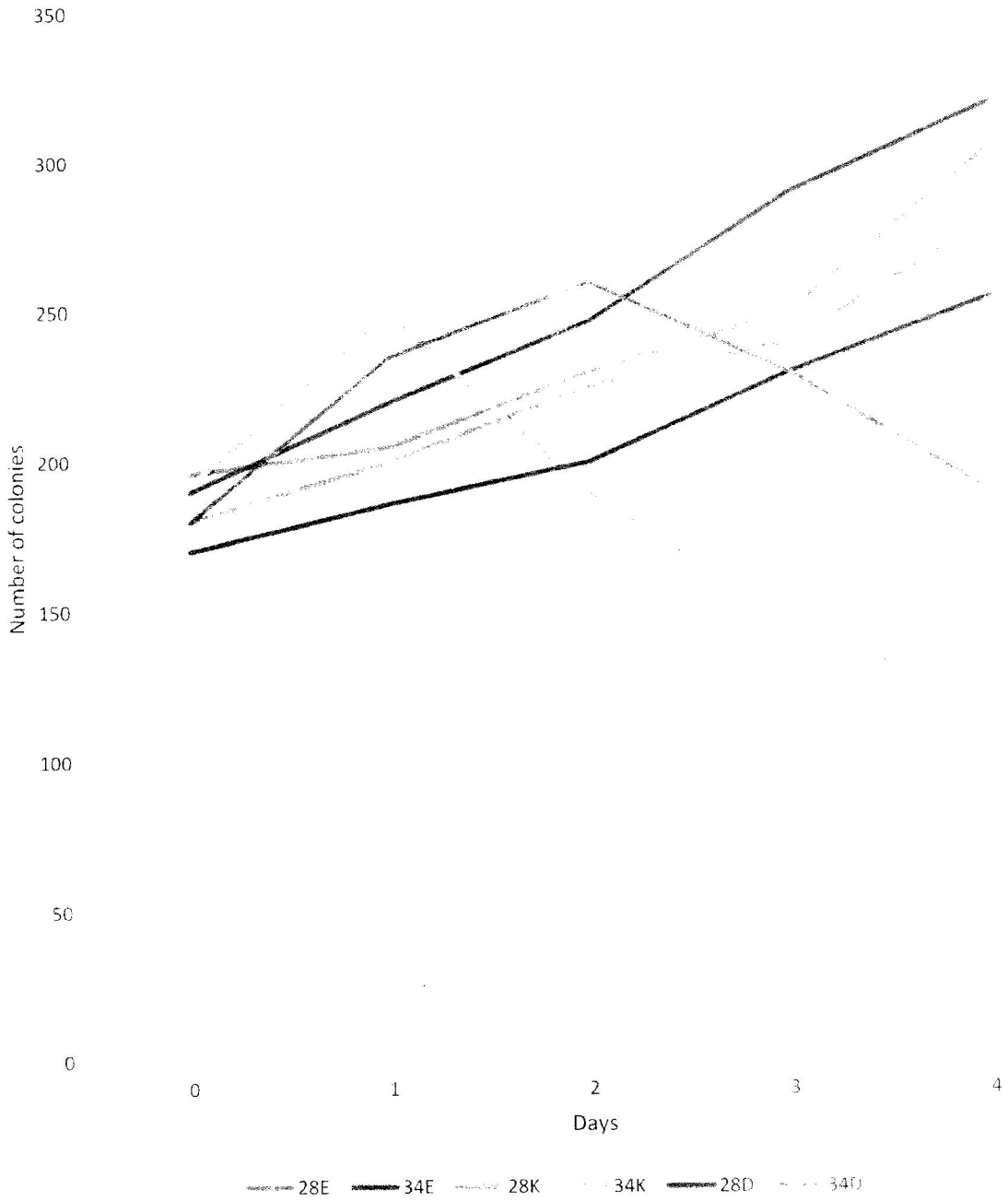


Figure 7: Total viable count of the isolates subjected to different carbon source

Keys:

E= Engine oil, K= Kerosene, D= Diesel, 28= *Pseudomonas* sp., 34= *Micrococcus* sp.

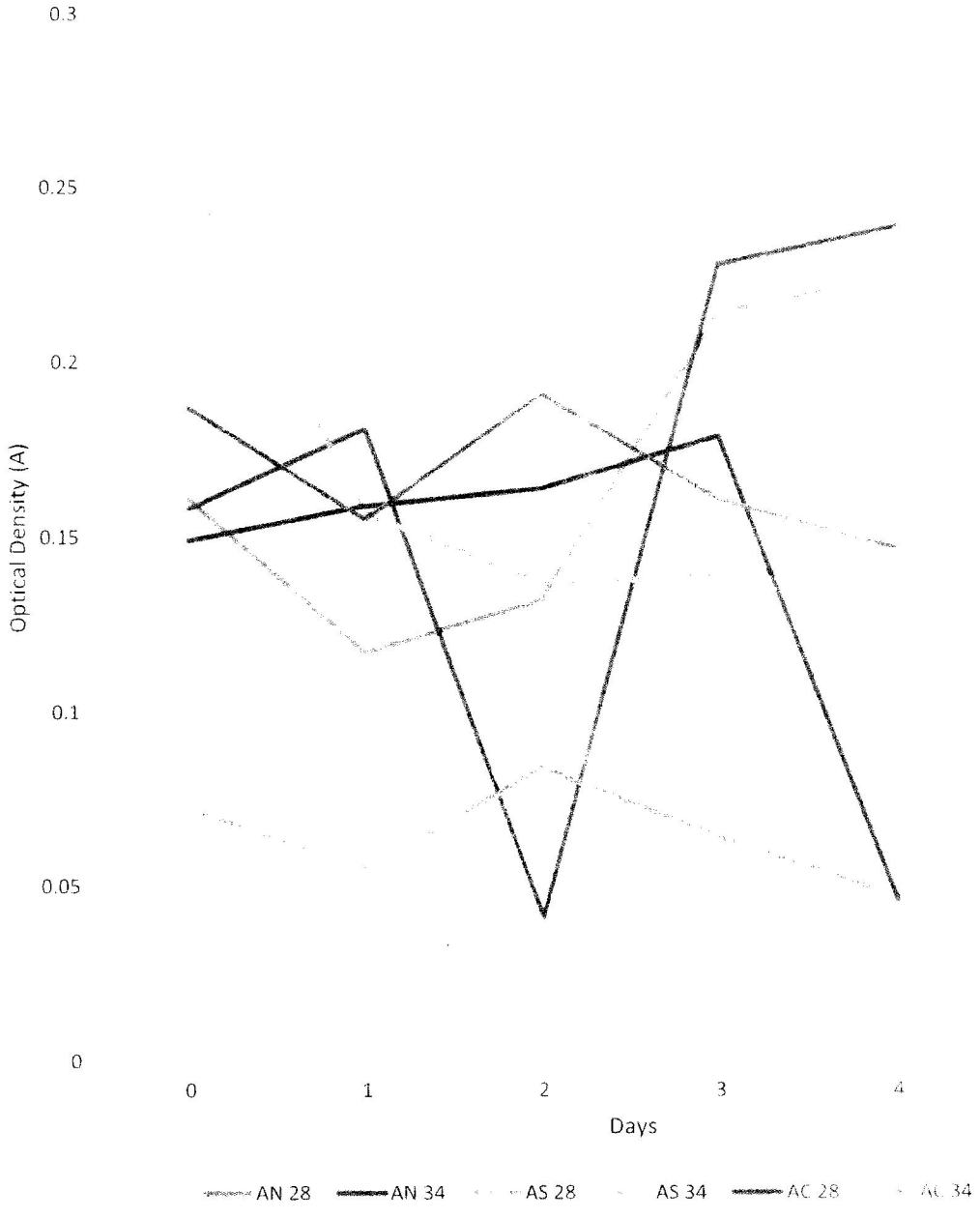


Figure 8: Optical density of bacteria subjected to different nitrogen sources

Keys:

AN= Ammonium nitrate, AS= Ammonium sulphate, AC= Ammonium chloride

28= *Pseudomonas* sp. 34= *Micrococcus* sp.

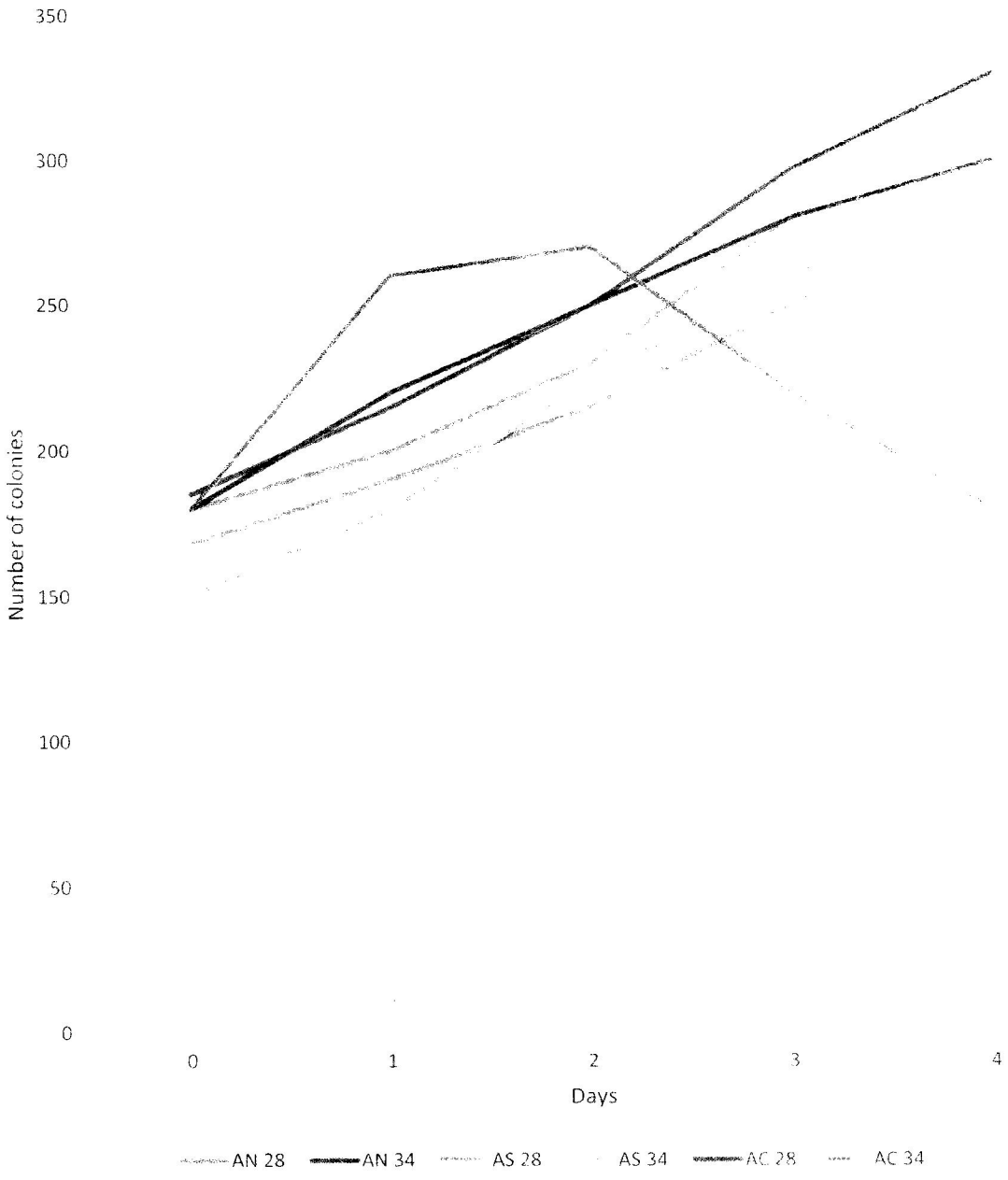


Figure 9: Total viable count of the isolates subjected to different nitrogen source

Keys:

AN= Ammonium nitrate, AS= Ammonium sulphate, AC= Ammonium chloride

28= *Pseudomonas* sp. 34= *Micrococcus* sp.

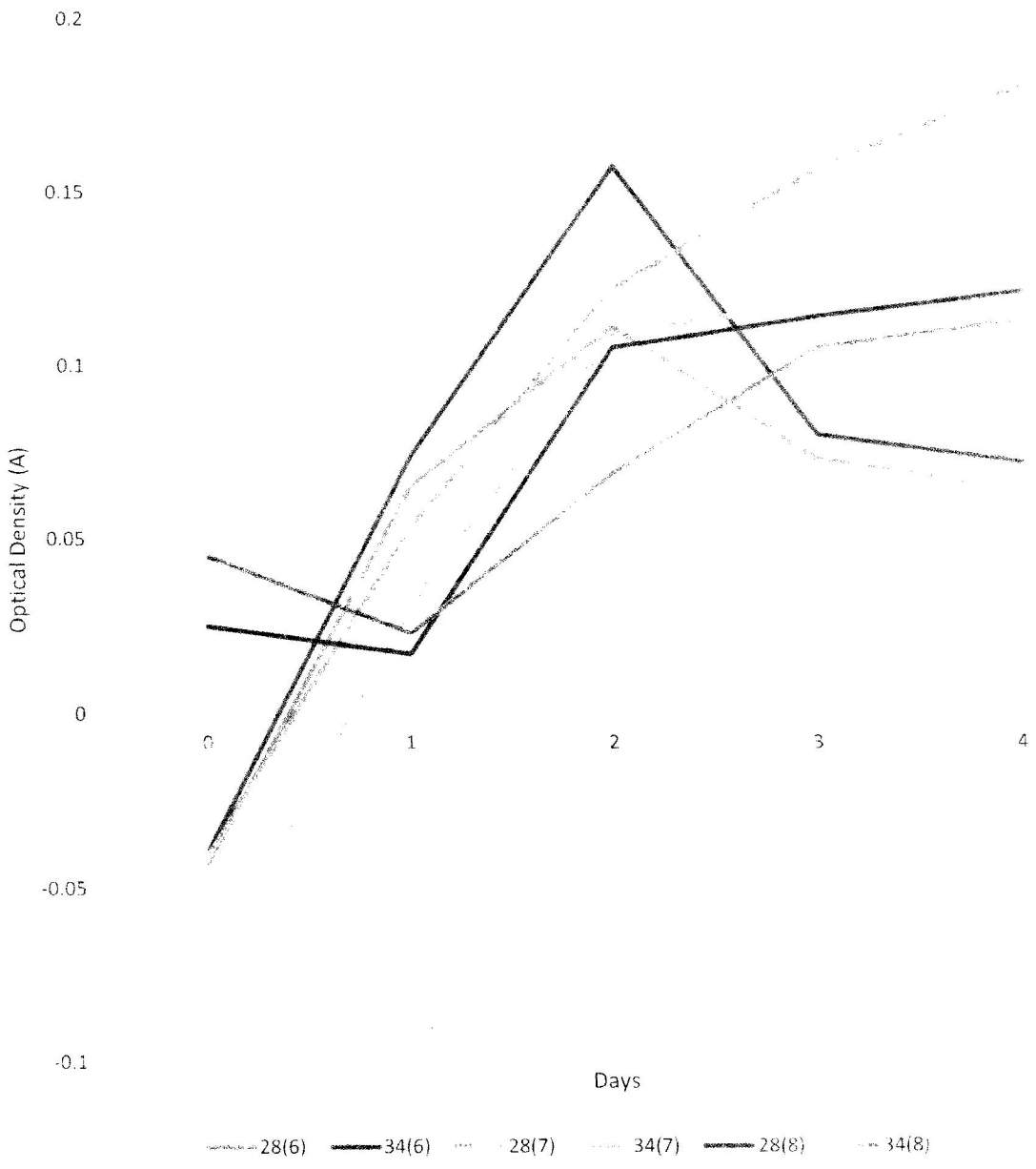


Figure 10: Optical density of bacteria subjected to different pH

Keys:

28= *Pseudomonas* sp., 34= *Micrococcus* sp., 6= pH 6, 7= pH 7, 8= pH 8

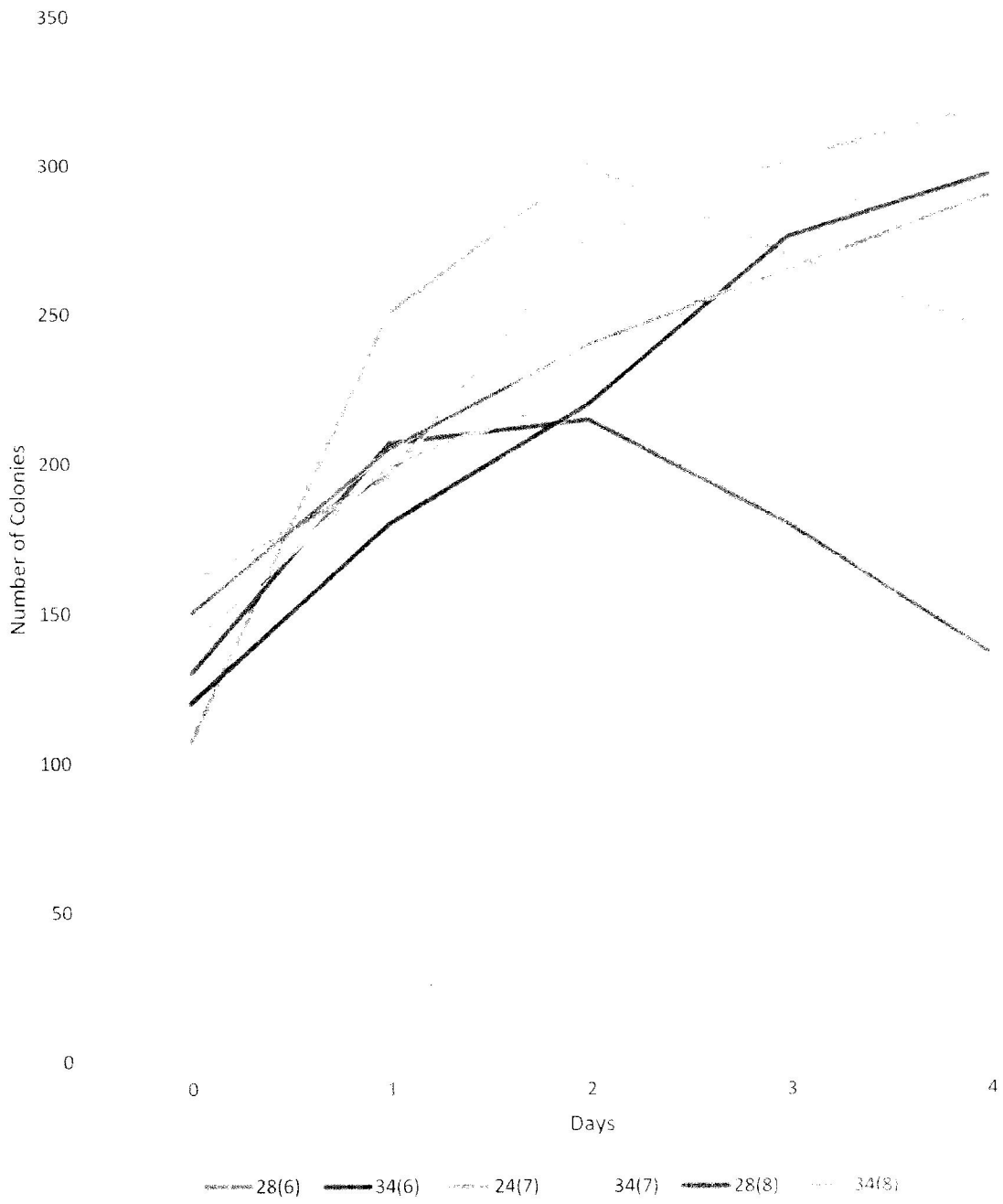


Figure 11: Total viable count of the isolates subjected to different pH

Keys:

28= *Pseudomonas* sp., 34= *Micrococcus* sp., 6= pH 6, 7= pH 7, 8= pH 8

CHAPTER FIVE

5.0. DISCUSSION/CONCLUSION

5.1. DISCUSSION

The world depend on oil. Vast amount of oil is used, transported, processed and stored around the world. Hydrocarbon compounds such as petroleum are essential for life. Since they do not naturally occur in the forms most useful to humans, they can be hazardous. Fuel and lubricating oil spills have become a major environmental hazard to date (Albaiges *et al.*, 2006).

Microorganisms which are said to be ubiquitous in nature are also found in oil contaminated soils. These microorganisms have the ability to use the contaminants as nutrient or energy sources (Tang *et al.*, 2012). Microorganisms degrade the hydrocarbon to produce carbondioxide and water. Several researchers have reported the occurrence of microorganisms that is capable of degrading hydrocarbons in the soil.

In this study, ten gram each of soils contaminated with used engine oil were collected from four different mechanic workshop in Oye-Ekiti and Ilupeju-Ekiti, Ekiti State (Plate 1). The samples were collected aseptically in a sterile polythene bag and immediately transported to the laboratory for studies. At each mechanic workshop the latitude, longitude and degree were observed in order to determine the location of the selected sites.

Since microorganisms are ubiquitous, numerous microorganisms are present in the soil. Heterotrophic bacteria are abundant in the soil since they can utilize the organic compounds in the soil as their energy and carbon source. The bacterial colony forming units (CFU) on the Nutrient agar for each mechanical workshops B, C, D & E were observed. Two diluent (diluent 3 and diluent 4) were cultured for each soil samples (Table 2). It was observed that the bacterial colony forming units (CFU) for each site, diluent 3 has the highest number of colonies compared to diluent 4. Sample D had the highest bacterial count for both diluent 3 and 4

($3.0x10^{-1}$ cfu/ml and $1.3x10^{-2}$ cfu/ml respectively) while sample E had the least bacterial count for both diluent 3 and 4 ($6.0x10^{-2}$ cfu/ml and $4.0x10^{-3}$ cfu/ml respectively). This is because more microorganisms were able to survive in soil sample D than sample E (Table 2). Ravi and Praveen, (2016) in their study reported that the bacterial colony forming units (CFU) of the four studied mechanical workshops A, B, C & D were 6×10^4, 7×10^4, 5×10^4 and 6×10^4 respectively.

Bioremediation is one of the most frequently used to remove hydrocarbons from the environment. In order to remove the hydrocarbon, different microorganisms, as well as consortia, are involved (Obayori *et al.,* 2009). Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon, microorganisms facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants (Johnsen *et al.,* 2005).

Thirty six bacteria were isolated from the soils of four mechanical workshops after culturing on Bushnell Hass Agar (BHA). Six bacteria were identified altogether, they include; *Bacillus* sp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus* sp., *Alcaligenes* sp., *Pseudomonas* sp. Fifteen bacteria were identified as *Staphylococcus aureus,* two were *Bacillus* sp., five were *Staphylococcus epidermidis*, four were *Pseudomonas* sp., six were *Micrococcus* sp., and four were *Alcaligenes* sp. (Table 3).

It was observed that in site B, that *Staphylococcus aureus,* and *Micrococcus* sp., were present. In site C, *Staphylococcus aureus, Pseudomonas* sp., and *Micrococcus* sp., were present. In site D, *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus* sp., *Alcaligenes* sp., and *Micrococcus* sp. were present. In site E, *Staphylococcus aureus, Staphylococcus epidermidis, Alcaligenes* sp., and *Pseudomonas* sp. were observed. *Staphylococcus aureus* was observed to be among the organisms present in all the sampled soil because it was isolated from all the sampled sites.

Pseudomonas sp., *Bacillus* sp., *Micrococcus* sp. and other bacterial strains was reported by Jesubunmi, (2014) in her study to be the hydrocarbon degraders obtained from spent engine oil contaminated soil taken from mechanic workshop along Opopo gbooro, Iworoko Road Ado Ekiti. Mandri and Lin (2007), Khan and Rizvi (2011) and Abioye *et al.* (2012), also isolated *Pseudomonas, Bacillus, Micrococcus* and other bacterial strains from engine oil contaminated soil.

Ravi and Praveen (2016), in their study isolated *Bacillus* species, *Acinetobacter* species, *Pseudomonas* species and *Micrococcus* species from four different mechanical workshops in Kaman area of Karimnagar town, Telangana state, India. Ogunbayo *et al.* (2012), isolated *Bacillus, Pseudomonas* and *Micrococcus* from oil contaminated sites of mechanical workshops located in Lagos city.

Shahida *et al.* (2015), isolated *Acinetobacter* species from oil contaminated sites of mechanical workshops in Sokoto metropolis. Manzoor *et al.* (2015), isolated fifteen bacterial species from oil contaminated soils of drilling oil site of Oil and Gas development company limited, Nashpa Karak and out of fifteen bacterial isolates seven were identified as *Bacillus* species.

The growth rate of the six identified bacteria were observed by measuring the optical density using the spectrophotometer at wavelength 600nm and total viable count by plating on Nutrient Agar. The spectrophotometer measures the turbidity or optical density which is the measure of the amount of light absorbed by bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells. The increase in the turbidity indicates increase in microbial cell mass. The total viable count help to know the number of organisms present in the broth culture. Increase in the number of colonies indicates increase in microbial cell mass (Swanson *et al.*, 1999).

Pseudomonas sp. and *Micrococcus* sp. has the highest growth rate compare to other organisms. From the chat (Figure 4&5), it was observed that from day 0 to day 4 there was increase in the number of colonies in both isolates compare to other organisms, this is because the two organisms were able to utilize the hydrocarbon which is used engine oil for their growth and metabolic activities. For other organisms (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Alcaligenes* sp.) had increase in optical density and number of colonies from day 0 to day 2 but decreased from day 2 to day 4. *Bacillus* sp. had increase in optical density and number of colonies from day 0 to day 3 but decreases from day 3 to day 4. (Figure 4 & 5). From day 0 to 2 there was growth of the organisms but from day 2 to 4 the organism had utilize all the nutrient in the broth which led to decrease in the number of colonies as a result of cell death.

The optimization of the growth conditions was carried out (by measuring the optical density using the spectrophotometer at wavelength 600nm and total viable count by plating on Nutrient Agar) on the bacterial isolate (*Micrococcus* sp. and *Pseudomonas* sp.) with the best growth rate. Carbon sources and Nitrogen sources were varied. Different carbon sources were used, which are; used engine oil, diesel and kerosene. It was observed in Figure 6&7 that *Micrococcus* sp. was able to utilize used engine oil as its carbon source compare to other carbon sources (diesel and kerosene) i.e. they can grow best in medium with used engine oil compare to diesel and kerosene. *Pseudomonas* sp. was able to utilize used engine oil and kerosene as carbon source compared to diesel. Therefore, used engine oil was the best carbon source for the two isolate (*Micrococcus* sp. and *Pseudomonas* sp.). Different nitrogen sources were used, which are ammonium nitrate, ammonium sulphate and ammonium chloride. It was observed in Figure 8&9 that the best nitrogen source for the growth of *Micrococcus* sp. and *Pseudomonas* sp. was ammonium nitrate while ammonium sulphate and ammonium chloride was the least nitrogen source. Mandri and Lin (2007), in their work varied different nitrogen

sources (ammonium nitrate, sodium nitrate, sodium nitrite and urea) in order to test the optimal conditions of each isolate (*Flavobacterium* sp., *Acinetobacter calcoaceticum* and *Pseudomonas aeruginosa*). Their result revealed that all the isolates were able to utilize ammonium nitrate as their carbon source. Different pH were used, which are 6, 7, and 8. It was observed in Figure 10&11 that the best pH for the growth of *Micrococcus* sp. and *Pseudomonas* sp. was pH 7 and 8 while pH 6 was the least pH. Mandri and Lin (2007), in their work also varied different pH (5, 7 and 9) in order to test the optimal conditions of each isolate (*Flavobacterium* sp., *Acinetobacter calcoaceticum* and *Pseudomonas aeruginosa*). The growth patterns were obtained by measuring the optical density at 600 nm and total viable counts (cfu/ml). Their result revealed that all the three strains showed maximum growth at pH 7 and above pH 7 the growth was declined.

5.2. CONCLUSION

In this study, six bacterial isolates were identified (*Bacillus* sp., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus* sp., *Alcaligenes* sp. and *Pseudomonas* sp.).The isolated bacterial species had grown by utilizing hydrocarbons present in the used engine oil as sole source of carbon. The most efficient oil degrading bacteria among the six isolated and identified bacteria were observed to be *Micrococcus* sp. and *Pseudomonas* sp. This work showed that bacteria are capable of degrading hydrocarbons, leading to environmentally-friendly products. Thus, these organisms when applied singly and as consortia can be used to clean up hydrocarbon-polluted environments.

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